Propeller Protein Containing a FYVE Domain, ProF: An Adaptor for Kinases Involved in Glucose Uptake in Adipocytes

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Table of contents

1 Summary

Adaptor proteins are essential components of many signal transduction pathways. Although not enzymatically active themselves, they can recruit important signaling components such as protein kinases to protein complexes or to specific subcellular locations in response to an activating signal. To perform this task, adaptor proteins often contain several different domains for coordinated protein-protein, protein-lipid, or protein-nucleic acid interaction.

In the first part of my thesis we identified and extensively characterized one potential adaptor protein. It contains two domains, a WD-repeat propeller and a FYVE domain, designated as ProF. We found that WD-repeats provided a protein- protein interaction platform, whereas the FYVE domain allowed interaction of ProF with internal vesicles. Two interaction partners of ProF were identified and investigated, the serine-threonine kinases Akt and PKCζ. ProF was found to preferentially interact with the activated kinases upon growth factor stimulation. We further demonstrated the interaction with ectopically expressed and endogenous proteins. Because activated Akt and the protein kinase C ζ, PKCζ, play an important role in insulin-stimulated translocation of the glucose transporter GLUT4 in adipocytes, we used adipocytes as a model system to study the function of ProF. We found that ProF translocated to the plasma membrane upon insulin stimulation, parallel to the kinases Akt2 and PKCζ/λ, and GLUT4. Overexpression of ProF led to increased glucose uptake, while knockdown of ProF by siRNA caused reduced glucose uptake. In summary, we concluded that ProF acts as an interaction partner of kinases and regulates glucose uptake into adipocytes.

Because ProF interacted with several proteins, we assumed that it could simultaneously bind to additional proteins. In that way, adaptor proteins can bring together kinases and their substrates. We searched for such substrate candidates and found in the second part of my thesis the membrane protein VAMP2 as interaction partner of ProF in vitro and in vivo. We further investigated the interaction of ProF, VAMP2, and PKCζ. We were able to show that these proteins colocalized on vesicular structures and demonstrated that ProF, VAMP2, and PKCζ form a ternary complex, in which ProF increases the binding of VAMP2 to PKCζ. Furthermore, we

found an IGF-1 stimulation- and PKCζ-dependent phosphorylation of VAMP2. Finally, we showed that ProF could recruit activated PKCζ to VAMP2 to increase phosphorylation of VAMP2 as substrate in vitro. These results further strengthened our hypothesis, that ProF can act as adaptor protein to integrate a kinase with its substrate. It further corroborated the assumption that ProF is involved in vesicle cycling, since VAMP2 is known to regulate vesicle docking and fusion.

The third part of my thesis focused on the question whether ProF, which plays a role in stimulation-dependent glucose uptake in adipocytes, also influences the generation of new adipocytes from undifferentiated pre-adipocytes, a process designated as adipogenesis. We found that knock down of ProF also influenced adipocyte differentiation, because protein and lipid droplet accumulation were delayed in adipocyte precursor cells. The effects of ProF knock down on protein accumulation and glucose uptake were limited to the first days of differentiation. ProF did not influence the growth rate of the cells, but influenced the expression of several adipocyte differentiation-related marker proteins. By using two different siRNAs targeted against ProF mRNA we found that complete downregulation of ProF strongly influenced glucose uptake and adipogenesis, whereas partial downregulation had much weaker effects. Together, these data indicate that ProF influences both, adipogenesis of pre-adipocytes and glucose uptake of adipocytes.

In conclusion, we characterized a new adaptor protein, which interacts with several proteins as demonstrated in pre-adipocytes and other cellular systems, such as brain tissue. Among these interaction partners are two kinases, Akt and PKCζ, and the protein VAMP2, which is involved in fusion of vesicles with their target membranes. Overexpression and knock down of ProF showed that it regulates differentiation of pre-adipocytes and glucose uptake in adipocytes. Furthermore, ProF may be more generally involved in a variety of vesicular trafficking processes, not only in adipocytes but also in other tissues and may bind to numerous other signaling proteins. Further research will shed light on the additional functions and interaction partners of this protein.

2 Zusammenfassung

Adapterproteine sind wesentliche Bestandteile zahlreicher Signalwege. Obwohl selbst nicht enzymatisch aktiv, können sie wichtige Proteine wie zum Beispiel Proteinkinasen nach deren Aktivation zu Proteinkomplexen oder zu bestimmten subzellulären Bereichen rekrutieren. Um diese Aufgabe zu erfüllen, besitzen Adapterproteine oftmals viele verschiedene Proteindomänen zur koordinierten Interaktion mit Proteinen, Lipiden oder Nukleinsäuren.

Der erste Teil meiner Arbeit behandelt die Identifizierung und ausführliche Beschreibung eines potentiellen Adapterproteins. Es beinhaltet zwei Proteindomänen, einen WD-repeat Propeller und eine FYVE Domäne, und wurde daher als ProF bezeichnet. Wir stellten fest, dass die WD-repeats für die Wechselwirkung mit anderen Proteinen verantwortlich sind, während die FYVE Domäne die Bindung von ProF an interne Vesikel ermöglicht. Zwei Bindungspartner von ProF, die Serin-Threonin Kinasen Akt und PKCζ wurden identifiziert und untersucht. Hierbei interagiert ProF bevorzugt mit den aktivierten Kinasen nach Wachstumsfaktor-Stimulation. Wir zeigten diese Bindung mit ektopisch überexprimierten und mit endogenen Proteinen. Da die aktivierten Kinasen Akt und PKCζ eine bedeutende Rolle in der insulinabhängigen Translokation des Glukosetransporters GLUT4 in Adipozyten spielen, benutzten wir Adipozyten als ein Modellsystem, um die biologische Funktion von ProF zu untersuchen. Wir stellten fest, dass ProF nach Insulin-Stimulation an die Plasmamembran translozierte, parallel mit den Kinasen Akt 2 und PKCζ/λ und mit GLUT4. Überexpression von ProF bewirkte eine erhöhte Glukoseaufnahme, während der Knockdown von ProF durch siRNA zu einer verminderten Glukoseaufnahme führte. Wir folgern daher, dass ProF die Glukoseaufnahme in Adipozyten reguliert und als Interaktionspartner von Kinasen fungiert.

Weil ProF mit verschiedenen Proteinen interagiert, vermuteten wir, dass es gleichzeitig mit weiteren Molekülen interagieren kann. Auf diese Weise können Adapterproteine Kinasen und deren Substrate zusammenführen. Im zweiten Teil meiner Arbeit suchten wir nach solchen potentiellen Substraten und entdeckten das Membranprotein VAMP2 als Interaktionspartner von ProF in vitro und in vivo. Wir

untersuchten die Interaktion zwischen ProF, VAMP2 und PKCζ genauer und konnten zeigen, dass diese drei Proteine auf vesikulären Strukturen kolokalisierten. Wir wiesen nach, dass ProF, VAMP2 und PKCζ einen Dreifach-Komplex ausbilden und dass ProF die Bindung von VAMP2 an PKCζ verstärkt. Des Weiteren stellten wir eine Phosphorylierung von VAMP2 fest, welche abhängig von IGF-1 Stimulation und PKCζ ist. Schliesslich zeigten wir, dass ProF aktiviertes PKCζ mit VAMP2 zusammenführen kann, um die VAMP2- Substratphosphorylierung in vitro zu verstärken. Diese Resultate bestätigten unsere Vermutung, dass ProF als Adapterprotein agiert, um eine Kinase und ihr Substrat zusammenzubringen. Zudem bekräftigten sie unsere Hypothese, dass ProF an vesikulären Prozessen beteiligt ist, da VAMP2 als ein Regulator solcher Prozesse gilt.

Der dritte Teil meiner Arbeit konzentrierte sich auf die Frage ob das Protein ProF, welches eine Rolle in der Insulin-abhängigen Glukoseaufnahme in Adipozyten spielt, auch einen Einfluss auf die Erzeugung neuer Adipozyten aus undifferenzierten Präadipozyten hat – ein Vorgang, der als Adipogenese bezeichnet wird. Wir stellten fest, dass der Knockdown von ProF in der Tat die Differenzierung der Adipozyten beeinflusst, da die Akkumulation von Protein und Fetttröpfchen in Zellen mit ProF Knockdown verzögert war. Die Auswirkungen von ProF Knockdown auf die Akkumulation von Protein und Fetttröpfchen waren auf die ersten Tage der Differenzierung beschränkt. Der Knockdown von ProF hatte keinen Einfluss auf die Wachstumsgeschwindigkeit der Zellen, sondern beeinflusste die Expression verschiedener für die Adipozyten-Differenzierung verantwortlicher Proteine. Indem wir zwei verschiedene siRNAs benutzten, die unterschiedliche Sequenzbereiche der ProF mRNA angriffen, stellten wir fest, dass eine vollständige Repression der ProF-Expression Glukoseaufnahme und Adipozyten- Differenzierung stark beeinflusste, während eine teilweise Repression viel geringere Effekte erzielte. Gesamthaft deuten diese Daten darauf hin, dass ProF sowohl den Prozess der Adipogenese von Präadipozyten als auch die Glukoseaufnahme in Adipozyten beeinflusst.

Zusammenfassend lässt sich sagen, dass ein neu entdecktes Adapterprotein charakterisiert wurde, dessen Interaktion mit verschiedenen Proteinen in Präadipozyten und anderen Zellsystemen wie Gehirngewebe demonstriert werden konnte. Unter diesen Interaktionspartnern sind zwei Kinasen, Akt und PKCζ, und das Protein VAMP2, welches an der Fusion von Vesikeln mit ihren Zielmembranen

beteiligt ist. Überexpression und Knockdown von ProF zeigten, dass das Protein die Differenzierung von Präadipozyten und Glukoseaufnahme in Adipozyten reguliert. Des Weiteren mag ProF eine allgemeinere Rolle in einer Vielzahl von Vesikeltransport- Prozessen haben – in Adipozyten wie auch in anderen Zellsystemen spielen und mit zahlreichen anderen Proteinen interagieren. Weitere Untersuchungen werden Aufschluss geben über die zusätzlichen Funktionen und Interaktionspartner von ProF.

3 Introduction

3.1 Signal transduction and adaptor proteins

A fundamental quest in biology is to understand the dynamic nature of the cell. The activity and subcellular localization of proteins within a cell is constantly modified in response to internal cues or external signals. These adjustments are performed by signaling molecules such as kinases and phosphatases. The regulation of these molecules strongly depends on interacting proteins and their binding to numerous signaling enzymes (Du and Tsichlis, 2005).

Protein-protein interaction can induce conformational changes to stabilize, destabilize, or alter the enzymatic properties of a signaling enzyme (Goh et al., 2004). Interacting proteins can also contribute to the formation of large protein complexes and thus allowing the coordinate response of multiple molecules to incoming signals (Stagljar, 2003). Finally, some proteins function as cellular anchors to recruit their binding partners to specific cellular structures. They may also facilitate the transport of their partners between cellular compartments (Fowler and Alarid, 2004).

To accomplish these diverse functions “adaptor proteins” have evolved. Adaptor proteins allow the formation of signaling complexes by binding their partner proteins either constitutively or in a signal-dependent manner (Kuriyan and Cowburn, 1997; Pawson and Scott, 1997).

Such adaptor proteins are important components of many signal transduction pathways. They usually are devoid of any intrinsic enzymatic activity themselves, but instead mediate specific protein-protein interactions, to relay important signaling events. Much of the specificity and effectiveness of signal transduction depends on the recruitment of signaling components such as protein kinases into short-lived active complexes in response to an activating signal such as binding of a growth factor to its receptor. A multitude of diverse interaction domains is found within adaptor proteins. These domains facilitate a rich diversity of specific and coordinated protein-protein, protein-lipid, or protein-nucleic acid interactions to occur within the cell during signal transduction. For example, while WD-repeats allow either stable or reversible binding to proteins and in some cases can also serve in phospho-peptide recognition of activated kinases, FYVE domains binds to phospholipids, present on

cellular membranes. Of special importance are so called “multidomain proteins”, in which several different modules cooperate to integrate diverse external or internal signals. In that way, they contribute to cellular responses such as changes in enzyme activity, gene expression or other activities.

One putative adaptor protein is a newly identified protein that was found by members of our institute as interacting protein of Akt - a serine-threonine kinase, which plays a key role in multiple signaling pathways. This newly identified protein contains seven WD-repeats, folding into a β-propeller, and a FYVE domain, and was designated as ProF. The multidomain structure of ProF with a protein-interacting module and a membrane-localization domain makes it an ideal adaptor protein- candidate. Thus, ProF may regulate external signals by recruiting key signaling molecules to distinct cellular locations. The characterization of ProF will be the topic of this thesis. Its structure, its interaction partners and the model system, in which a possible physiological role of ProF was studied, will be addressed in this introduction.

In the first part, the domains of ProF - WD-repeats and FYVE domain - will be discussed in more detail. I will describe their structure and function with special emphasis on signal transduction.

In the second part, I will introduce the interacting partners of ProF, the serine-threonine kinase Akt, as well as the atypical protein kinase C isoform PKCζ, which was also found as a binding partner of ProF and further investigated during this thesis.

In the third part, the insulin-dependent glucose uptake in adipocytes will be applied as a model system in which the two interaction partners of ProF, Akt and PKCζ, play a key role.

3.2 WD-repeat proteins

3.2.1 Distribution and structure of WD-repeats

WD-repeat proteins, also known as WD40-repeat or Trp-Asp repeat proteins, form a large and structurally highly conserved protein family in eukaryotes. Only recently, WD-repeat proteins have been found in prokaryotes (Stoytcheva et al., 2000), in which, however, they appear to be extremely rare (Madrona and Wilson, 2004). WD-repeats are widely distributed in many hundreds of eukaryotic proteins, comprising a significant percentage of their proteome. Each WD-repeat contains about 40 amino acids, hence the synonym WD40-repeat, but the total number of amino acids can rise up to 60 amino acids per repeat (Anderson and Parker, 1998). The name WD-repeat is derived from a weakly conserved Trp (W)-Asp (D) dipeptide found at the C-terminal end of the consensus sequence. Typically, a Gly (G)-His (H) dipeptide is found between the N-terminus and the WD-dipeptide. However, neither the GH nor the WD motif is absolutely conserved, and the whole WD-motif shows very limited amino acid sequence conservation (Neer et al., 1994). WD-repeats within a polypeptide are usually not more similar to each other than they are to repeats in other WD-repeat proteins. Furthermore, the sequences can contain large insertions, which complicate their identification on the basis of sequence information alone.

Known WD-repeat proteins range in size from small proteins (ca. 33 kDa) such as the pleiotropic plant developmental regulator VIP3 (Zhang et al., 2003) to large (>400-kDa) proteins such as the mammalian protein trafficking factor Lyst (van Nocker and Ludwig, 2003). WD-repeat containing proteins mostly have four – eight copies of the WD-repeat motif, but up to 16 copies can be found in a single protein (Smith et al., 1999): see GenBank D63999).
The WD-repeat propeller structure creates a stable and probably very rigid platform for protein interactions. Up to date (2006) seven WD-repeat containing proteins were analyzed by X-ray crystallization and structure determination:

- The β-subunit of the cell-signaling G protein transducin (Sondek et al., 1996; Wall et al., 1995).
- A fragment of the Tup1p corepressor protein (Sprague et al., 2000),

- The p40 subunit of the Arp2/3 complex (Robinson et al., 2001),
- A domain of the Groucho/TLE1 corepressor protein (Pickles et al., 2002)
- The actin-interacting protein Aip1p, a regulator of depolymerization of actin filaments (Voegtli et al., 2003),
- The small protein Ski8p, a regulator of degradation of damaged mRNA (Cheng et al., 2004; Madrona and Wilson, 2004),
- Sif2p, a component of the Set9 complex (SET3C), responsible for the repression of a set of meiotic genes (Cerna and Wilson, 2005).

Taken together, these data allow several conclusions about the general structure and function of WD-repeat proteins. Seven WD-repeats form a highly symmetric β-propeller structure. The only exception known to date is the structure of Sif2p, which revealed an eight-bladed propeller (Cerna and Wilson, 2005). It has been speculated for a long time whether proteins with more than eight WD domains will form one big propeller or two small ones. In the case of Aip1p, a WD-repeat protein that regulates cofilin-mediated depolymerization of actin filaments (Konzok et al., 1999; Ono, 2001) X-ray crystallization and structure analysis was performed and the protein was found to contain 14 WD-repeats, which fold into two connected seven-bladed β-propellers (Voegtli et al., 2003).

Each WD-repeat is made up of a small antiparallel β-sheet consisting of four short β-strands (Fig.1). These strands are labeled A, B, C and D by convention, beginning with the strand closest to the center and ending with the outside strand. However, one repeat in the amino acid sequence does not coincide with one structural WD-repeat propeller blade. The order of the strands in the WD-repeat amino acid sequence is D-A-B-C, such that one sequence repeat composes the D strand of one blade and the A, B, C strand of the succeeding blade, causing an overlapping structure (Fig.1). This structural feature is conserved among all β-propeller proteins and is believed to be very important for protein stability (Jawad and Paoli, 2002; Neer et al., 1994).

The loops connecting the individual strands are designated by the two strands they connect (e.g. the A-B loop connects strands A and B). By convention, the “top” of the protein is defined by the surface that contains the D-A and B-C loop. In general, the

existence of well-ordered blades with large or disordered D-A loops and C-D turns suggest that it is possible to maintain the blade structure despite large variations in the traditional WD-repeat pattern. Protein-protein interactions with WD-repeat proteins are often mediated by contacts with the variable regions composed of the "top", "bottom" and, to a lesser extend, sides of the protein. It is assumed that the binding of interacting proteins to exposed loop- like regions of WD-repeat proteins would allow modulation of protein-binding properties without significant perturbation of the core structure.

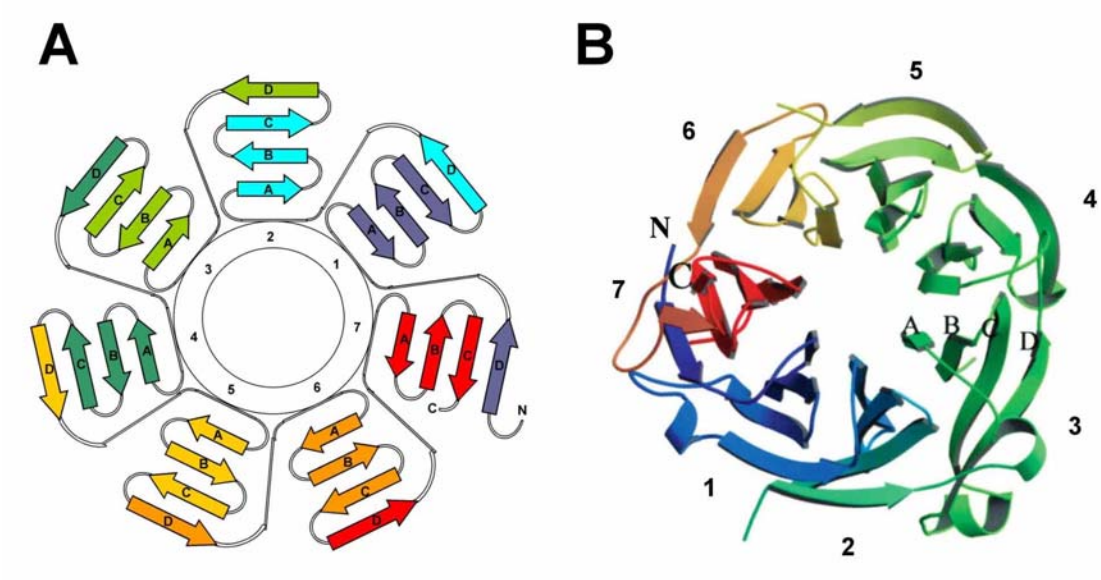

Fig. 1A: Schematic model of a WD-repeat propeller, containing seven propeller blades. Each of the blades (numbered 1 – 7 in the central circle) is formed by a small anti-parallel β-sheet consisting of four short β-strands, indicated as arrows. The strands are labeled A, B, C, and D, beginning with the strand, which is closest to the center of the propeller. Note that a single WD-sequence repeat (indicated in the structure as arrows of identical color) comprises the D strand of one blade and A, B, and C strand of the following blade. Therefore, one repeat in sequence does not coincide with one structural repeat or blade. For further details see text.

Fig. 1B: Crystal structure of the yeast protein Ski8p, a regulator of mRNA degradation, as determined by (Cheng et al., 2004). Ski8p shows the characteristical seven-bladed β-propeller structure of a WD-repeat protein, as depicted in a ribbon diagram. Each blade consists of a four-stranded β-sheet (labeled A–D to the right). The view is looking down the central axis of the propeller onto the "top" surface, defined by the presence of the D–A loops connecting sequential blades. The N- and C-terminus of the protein are denoted to the left. For further details see text.

Structures of the Gβ protein (Loew et al., 1998; Wall et al., 1995), p40 (Robinson et al., 2001), and Cdc4 (Orlicky et al., 2003) in complex with partner proteins or peptides demonstrate full or partial involvement of the loops in binding. In the case of the WD-repeat protein β-TrCP1 is has been shown that all seven WD-repeats

contribute to contacts to β-catenin. Two thirds of the contacts involve the A strand and the rest involves B-C loops (Wu et al., 2003).

3.2.2 Function of WD-repeat proteins

WD-repeats do not possess any catalytic activity (Neer et al., 1994). Instead, WD-repeat proteins form a recognition module for linking partner proteins in intracellular networks of signaling and sorting. The common function of all WD-repeat proteins is the coordination of complex multiprotein assembly in an multitude of cellular systems (Li and Roberts, 2001; Smith et al., 1999) by specifically binding to one or more partner proteins. This is often done to present a protein substrate molecule to an enzyme such as a kinase or ubiquitin ligase.

WD-repeat proteins are involved in a diverse range of physiological processes, which can be roughly categorized into ten groups (modified after (Neer et al., 1994; van Nocker and Ludwig, 2003; Yu et al., 2000)):

- Signal transduction (Clapham and Neer, 1997; McCahill et al., 2002)
- RNA synthesis, degradation and processing such as splicing and export (Harnpicharnchai et al., 2001; Ohnacker et al., 2000; Saveanu et al., 2003)
- Chromosomal modifications such as chromatin assembly (Martinez-Balbas et al., 1998; Tyler et al., 1996).
- Vesicular trafficking (Rybakin and Clemen, 2005).
- Transcriptional regulation and repression (Chen and Courey, 2000).
- Protein degradation (Brunson et al., 2005).
- Cytoskeletal assembly and dynamics (Voegtli et al., 2003).
- Cell cycle control and regulation, such as mitotic spindle formation (Li and Roberts, 2001; Smith et al., 1999).
- Apoptosis (Benedict et al., 2000; Lauber et al., 2001).
- Unknown functions

Because mediation of signal transduction is an important aspect of the function of WD-repeat proteins in general and of the protein characterized in this thesis in particular, I will now focus mainly on the role of WD-repeat proteins in signal transduction.

3.2.3 WD-repeat proteins and signal transduction

A growing number of WD-repeat proteins is known to be involved in signal transduction. For brevity, I will restrict this introduction to the best characterized proteins, the receptors for activated C kinases (RACKs).

3.2.3.1 RACK proteins

RACK proteins, which are entirely made up of WD-repeats are required for the function of protein kinase C (PKC). Up to now, two RACK proteins have been identified, RACK1, a binding partner for activated PKCβII and RACK2, a binding partner for activated PKCε.

3.2.3.1.1 RACK1

RACK1 is a cytosolic 36-kDa protein, which is highly conserved in animals and plants (McCahill et al., 2002). As member of the WD-repeat family of proteins, RACK1 is predicted to fold into a β-propeller-like structure, comprising seven WD-repeats (Ron et al., 1994). These findings are in agreement with data recently obtained from cryo-electron microscopy (Sengupta et al., 2004).

RACK1 has several independent protein-binding sites and can simultaneously interact with a number of signaling molecules (Rodriguez et al., 1999). This allows RACK1 to integrate inputs from distinct signaling pathways.

RACK1 interacts selectively and saturable with activated PKCβII (Ron et al., 1994) by binding to the C2 domain of PKCβII. RACK1 also shuttles activated PKCβII to its correct cellular localization (Ron et al., 1999). The antagonist peptide βC2-4, which corresponds to amino acids 218 –226 of PKCβII, can compete with PKCβII for binding and inhibits phorbol ester-induced translocation of PKCβII in cardiac myocytes, and insulin-induced PKCβII translocation and function in Xenopus oocytes (Ron et al., 1995). RACK1 does not appear to be a PKC substrate itself, but facilitates the interaction of PKCβII with its substrates and thus increases PKCβII substrate phosphorylation by several-fold (Schechtman and Mochly-Rosen, 2001).

Recently it has been found that RACK1 also binds to activated PKCδ (Rosdahl et al., 2002) and PKCμ (Hermanto et al., 2002). Besides activated isozymes of PKC

RACK1 has many other binding partners, including β1, β2, β3, and β5 integrins (Buensuceso et al., 2001; Liliental and Chang, 1998), the common β-chain of the interleukin-5 (IL-5) receptor (Geijsen et al., 1999), phospholipase C-γ (Chang et al., 2001), β-spectrin and dynamin (Rodriguez et al., 1999); the protein tyrosine phosphatase PTPμ (Mourton et al., 2001), the Src- kinase (Chang et al., 2001; Chang et al., 1998; Chang et al., 2002a; Miller et al., 2004), and a phosphodiesterase isoform (Yarwood et al., 1999). Some of these interactions are mutually exclusive and some are concurrent. This suggests that RACK1 may act as a scaffolding protein, recruiting proteins to various transmembrane receptors and providing a platform for protein–protein interactions. Much remains unknown concerning the functional role of RACK1; however, in vivo studies have indicated that RACK1 is upregulated in human carcinomas and during tissue regeneration after ischemic renal injury (Berns et al., 2000; Padanilam and Hammerman, 1997), suggesting a role of RACK1 in proto-oncogenic signaling events.

3.2.3.1.2 RACK2

RACK2, also known as β'-coatomer protein (COP), is a part of the coatomer complex (Harrison-Lavoie et al., 1993; Stenbeck et al., 1993). It binds to activated PKCε selectively and saturable (Csukai et al., 1997a; Csukai et al., 1997b). It is not a PKC substrate itself, but increases PKCε substrate phosphorylation by several-fold and is likely to be involved in vesicular release and cell- cell communication.

These findings of a second receptor for activated C kinases led to the assumption that PKC isoform specificity is guided by interactions with a family of RACK proteins. Those proteins display specific and saturable binding only to the activated conformation of PKC isoforms and act as targeting signals (Mochly-Rosen and Gordon, 1998). More generally, other WD-repeat proteins could, by preferentially binding to activated kinases, play a key role in signal transduction.

3.2.4 Additional functional domains of WD-repeat proteins

WD-repeat containing proteins often have additional functional domains. Some of these additional motifs are zinc-binding motifs, actin-binding motifs, F-boxes, leucine

zippers, (Src homology-3) SH3 domains binding to proline rich sequences, BROMO domains as found in chromatin-associated proteins, or the catalytic domains of kinases. Such associated protein domains can determine the subcellular localization of the protein by binding to specific compartments. There the WD-repeats can bring together interacting partners at their correct site of action.

3.3 FYVE domains

Numerous cellular processes such as signal transduction, vesicle trafficking, and cytoskeletal rearrangement require the targeting of proteins to subcellular membranes. Membrane targeting is largely achieved by specific recognition of particular membrane lipids by proteins. A diverse group of such membrane-targeting domains, which specifically recognize different types of membrane lipids, have been identified. One of these motifs is the FYVE domain, which shows high specificity and affinity exclusively for phosphatidylinositol 3-phosphate (PI3P) and will be discussed in detail.

3.3.1 Sequence and structure of FYVE domains

FYVE domains are short (70-80 residues long) sequence motifs named after the four proteins **F**ab1p, **Y**OTB, **V**ac1p, and **E**EA1, in which this domain was originally observed (Stenmark et al., 1996). Database searches revealed that the human proteome contains 27 FYVE proteins (Stenmark et al., 2002). For *Caenorhabditis elegans* 13 FYVE proteins were reported, while in *Saccharomyces cerevisiae* 5 FYVE domains were identified (Stenmark et al., 1996).

FYVE domains contain eight conserved zinc-coordinating cysteines (Stenmark et al., 2002) to form a zinc-finger like structure (Misra and Hurley, 1999) and a highly conserved, basic (R/K)-(R/K)-H-H-C-R sequence surrounding their third cysteine (Fig. 2). The crystal structures of FYVE domains revealed their great similarity to the C1 domain of PKC and the cysteine-rich region of rabphilin 3A (Kutateladze et al., 1999; Misra and Hurley, 1999). FYVE domains additionally contain a long loop at the N-terminus, two short antiparallel ß-sheets (ß-2 and ß-3) and a C-terminal α-helix, α-1, as seen in Fig. 2. The PI3P-binding site is formed by the basic residues in the conserved (R/K)-(R/K)-H-H-C-R sequence, which fold into a shallow, positively

charged pocket that dominates the surface of the FYVE domain. Side-chains from this motif largely account for the binding between the PI3P head group and the FYVE domain as demonstrated in the case of the FYVE-protein EEA1 crystallized in complex with phosphoinositide (Dumas et al., 2001). Importantly, the structure furthermore contains a prominent hydrophobic protrusion (Fig. 2) that allows the domain to penetrate the membrane bilayer (Misra and Hurley, 1999).

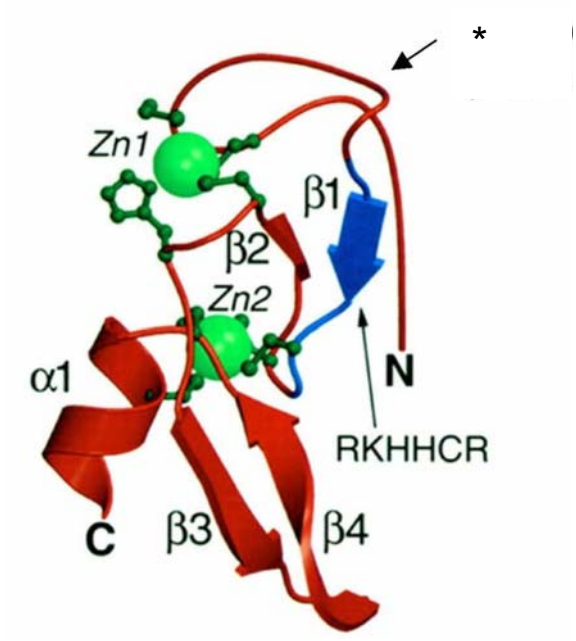

Fig. 2: The crystal structure of the FYVE domain of the yeast protein vsp27p is depicted, as determined by (Misra and Hurley, 1999) and discussed by (Corvera, 2000). The cysteine residues and a single histidine residue involved in the coordination of two Zn^{++} atoms (Zn1 and Zn2 in light green) are highlighted in green. The highly conserved positive residues in the ß1 strand, involved in PI3P binding, are depicted in blue. The amino acids that comprise the other three ß-sheets (ß2 - ß-4) and the C-terminal α1-helix are depicted by red arrows and a red helix (left). The hydrophobic protrusion that allows membrane penetration is indicated to the top right. For further details see text.

It is important to note that, because of the relatively shallow PI3P-binding pocket and a single phosphate interaction, isolated FYVE domains are in the most cases insufficient for membrane localization and require adjacent sequences that interact with other proteins or allow dimerization for efficient membrane recruitment (Dumas et al., 2001; Lawe et al., 2000). However, there is a small number of FYVE-containing proteins that have been reported to autonomously translocate to endosomal membranes when expressed ectopically (Ridley et al., 2001; Seet and Hong, 2001; Tsukazaki et al., 1998). The increased endosomal targeting properties of these proteins can be partly attributed to their unique structure. The FYVE domain of endofin has a strongly positively charged membrane-binding surface, which greatly enhances its affinity for PI3P-containing vesicles (Blatner et al., 2004). Another highly interesting FYVE-containing protein, which will be dealt with in greater detail below, is the FYVE-domain containing protein localized to endosomes-1 (FENS-1). The structural feature that determines the high membrane affinity of the FYVE

domain of FENS-1 is a unique 11-amino acid insertion next to the conserved (R/K)-(R/K-)H-H-C-R sequence. This motif forms an extended turret loop rich in aromatic side chains, resulting in a high membrane penetration power (Blatner et al., 2004).

3.3.2 FYVE binding to phosphoinositides

FYVE domains localize several proteins that participate in vacuolar sorting or endocytosis to membranes by interacting with PI3P (Burd and Emr, 1998; Simonsen et al., 1998; Stenmark et al., 1996). PI3P are generated from phosphatidylinositol by specific isoforms of phosphatidylinositol 3-kinase (PI3K). Even though the levels of PI3P within the cell are low, they play a key role in eukaryotic membrane trafficking and are found in specific subcellular localizations, including the cytoplasmic face of early endosomes and internal vesicles of multivesicular bodies (Stenmark and Gillooly, 2001). A large body of work has shown that PI3P is involved in the regulation of trafficking in the early endocytic pathway (Corvera, 2000) as well as in Golgi/ vacuole sorting (Odorizzi et al., 1998) and appears to serve as a specific marker for a subset of endosomes, the “early endosomes”. Only recently, it has been found that PI3P is also generated in a stimulus-dependent fashion (Razzini et al., 2000; Vieira et al., 2001; Zhang et al., 1998), including insulin-mediated formation of PI3P at lipid raft subdomains of the plasma membrane (Maffucci et al., 2003).

3.3.3 The functions of FYVE proteins

3.3.3.1 FYVE domain proteins involved in membrane trafficking

The most extensively studied FYVE domain protein is the early endosome antigen 1 (EEA1), which serves as a good example for the main function of FYVE proteins, the regulation of membrane trafficking.

EEA1 is a large coiled-coiled protein with a C-terminal FYVE domain (Mu et al., 1995). This protein also contains two binding domains for the early-endosomal Rab5 in its active, GTP-bound state (Simonsen et al., 1998). Endosomal targeting of EEA1 requires both binding to PI3P and to Rab5-GTP. It has been proposed that this dual binding requirement enables the very specific and regulated recruitment of EEA1 to early endosomes (Lawe et al., 2000; Patki et al., 1997; Simonsen et al., 1998).

EEA1 plays a key role in endosome fusion, since depletion of EEA1 inhibits, and excess of EEA1 stimulates, this process *in vitro* (Christoforidis et al., 1999; Simonsen et al., 1998). Endosome fusion also requires Rab5-GTP, which has to be present on both membranes (Barbieri et al., 1998). Thus, EEA1 likely acts as a tether for two Rab5-positive membranes (Rothman, 1994), and thereby drives membrane fusion. The role of the FYVE domain and its interaction with PI3P is probably to concentrate EEA1 at the correct subcellular location to participate in this process.

Another FYVE-domain-containing protein, which has been implicated in vesicular trafficking, is Fab1p from yeast. This protein is a PI3P 5-kinase, i.e. it phosphorylates PI3P to form phosphatidylinositol-(3,4)-biphosphate ($PI(3,5)P_2$) (McEwen et al., 1999). $PI(3,5)P_2$ is a comparatively recently discovered phosphoinositide (Cooke et al., 1998), for which there is at present no known effector. *fab1* mutants have morphological defects such as expanded vacuoles, indicating a role for $PI(3,5)P_2$ in vacuolar homoeostasis in yeast (Odorizzi et al., 1998). A mammalian ortholog of Fab1p, termed PI3P 5-kinase containing a FYVE domain (PIKfyve) has been identified (Sbrissa et al., 1999). This protein is able to produce both PtdIns5P and $PI(3,5)P_2$ *in vitro* and has been reported to play a role in the trafficking between the late endosome and the lysosome (Ikonomov et al., 2001; Shisheva, 2001). More recently it has been shown to be phosphorylated in 3T3-L1 adipocytes by the serine-threonine kinase Akt in response to insulin (Berwick et al., 2004).

3.3.3.2 FYVE domain proteins involved in signal transduction

An example of a FYVE finger protein that is involved in a signaling cascade is the Smad anchor for receptor activation, (SARA). This protein is responsible for the recruitment of Smad2 and Smad3 to the transforming growth factor (TGF)-β receptor upon receptor stimulation (Tsukazaki et al., 1998). These Smad proteins are then phosphorylated and activated by the activated receptor kinase. The phosphorylation of Smad2 and Smad3 enables them to bind Smad4 and to translocate to the nucleus, where the complex controls the transcription of target genes. Since SARA is located on early endosomes by binding to PI3P the role of SARA in the TGF-β signaling cascade is probably to recruit molecules to early endosomes, to which the receptor–ligand complex localizes upon endocytosis. SARA thus provides a potential example of signaling not from the plasma membrane but from the endosomal

membrane (Ceresa and Schmid, 2000). It also represents an example of a role for a FYVE domain containing protein in a cell-signaling cascade.

3.4 The serine-threonine kinases Akt and PKCζ/λ

3.4.1 Akt

3.4.1.1 History of Akt

The serine-threonine Akt was initially identified as an oncogene transduced by the AKT8 acute transforming retrovirus, which was isolated from an AKR mouse thymoma in 1977, hence its name. This virus induced malignant transformation in the mink lung epithelial cell line CCL-64 and tumor formation, specifically thymic lymphoma, in nude mice (Staal et al., 1977). A decade later, its defective retrovirus was identified from mink lung epithelial cells infected with AKT8 virus, and was shown to contain a cell-derived protooncogenic sequence, which was termed Akt (Staal and Hartley, 1988; Staal et al., 1988).

In the early 1990's, sequence analysis of the Akt viral oncogene and its cellular homolog revealed that it encodes a serine-threonine protein kinase, composed of a N-terminal pleckstrin homology (PH) domain and a C-terminal kinase domain very similar to that of PKC and PKA (Bellacosa et al., 1991). At the same time, Akt was also cloned based on its homology with PKC or PKA by two additional groups, who named it RAC (protein related to protein kinase A and protein kinase C) or PKB (protein kinase B) (Coffer and Woodgett, 1991; Jones et al., 1991). To date, the protein is most commonly named Akt/PKB, and will be referred to as Akt here.

3.4.1.2 Conservation of Akt

The Akt family of kinases is evolutionarily conserved in eukaryotes. Orthologs of Akt were found in and cloned from a number of species including *Drosophila* and *C. elegans*, demonstrating a wide evolutionary conservation (Franke et al., 1994). Homologs were also found in the slime mould *Dictyostelium* (Meili et al., 1999) and a protein isoform lacking the N-terminal PH domain was identified in *Saccharomyces cerevisiae* (Jorgensen et al., 2004). The amino acid identity between *C. elegans* and human Akt is around 60%, whereas that between mouse, rat and human it is more than 95%. Akt shares a similarity in its catalytic domain with a group of kinases from the large PKA, PKG, and PKC (AGC) superfamily of serine-threonine kinases that consists of more than 80 kinases.

3.4.1.3 Isoforms of Akt

Three major isoforms of Akt, encoded by three separate genes, have been identified in mammalian cells (Fig. 3). Akt1 and Akt2 were the first isolated isoforms (Bellacosa et al., 1991; Cheng et al., 1992; Coffer and Woodgett, 1991; Jones et al., 1991). Akt3 was subsequently cloned through homology screening (Konishi et al., 1995). While Akt1 is the true human homolog of the v-akt, originally identified by Staal and coworkers (98% identity at the amino acid level), Akt2 and Akt3 are v-akt closely related kinases (Cheng et al., 1992; Nakatani et al., 1999a). The overall sequence identity between these three Akt- isoforms is >85%. They share a very similar structure and possess conserved threonine and serine residues (T308/S473 in Akt1, T309/S474 in Akt2 and T305/S472 in Akt3) that together with the PH domain are critical for Akt activation (Fig.3).

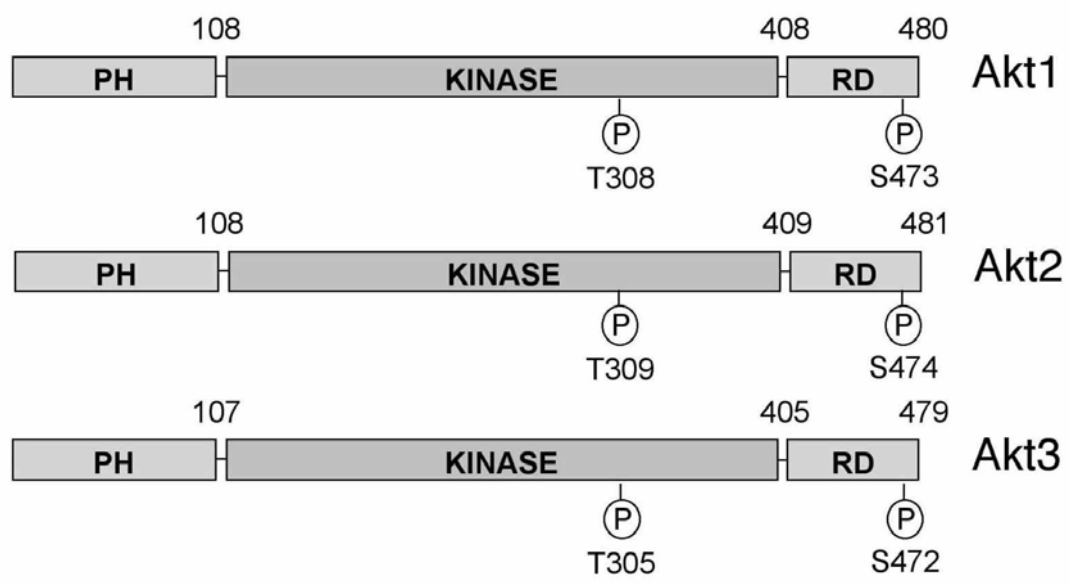

Fig. 3: The three mammalian Akt isoforms Akt1, Akt2, and Akt3 are depicted. All isoforms share a highly similar structure with a N-terminal PH domain for binding to PIP_3, a central kinase domain (KINASE) and a C-terminal regulatory domain (RD). The essential threonine and serine residues necessary for activation are indicated. For further details see text.

Although Akt1, Akt2, and Akt3 display high sequence homology, there are clear differences between them in terms of biological and physiological function: First, overexpression of wild type-Akt2, but not Akt1 and Akt3, transforms NIH 3T3 cells and induces invasion and metastasis in human breast and ovarian cancer cells (Arboleda et al., 2003; Cheng et al., 1997); second, the gene *Akt2*, but not *Akt1* and

Akt3, is frequently amplified in certain types of human cancer even though alterations of all three isoforms of Akt have been detected in human malignancies (Bellacosa et al., 1995; Cheng et al., 1992; Cheng et al., 1996; Miwa et al., 1996; Nakatani et al., 1999b; Ruggeri et al., 1998; Yuan et al., 2000); third, *Akt1* expression is relatively uniform in various normal organs. High levels of *Akt2* mRNA are also detected in various tissues, usually at a lower level than Akt1 except in insulin- responsive tissues, such as fat cells, liver and skeletal muscles. Akt3 is expressed at the lowest level in most adult tissues except testes and brain (Brodbeck et al., 1999; Nakatani et al., 1999a). Forth, Akt2, but not Akt1, plays an unique role in muscle differentiation (Kaneko et al., 2002) and fifth, *Akt1*-, *Akt2*- and *Akt3*-deficient mice displayed different phenotypes. Shortly, *Akt1*-/- knockout mice were found to be small and had increased spontaneous apoptosis in the thymus (Chen et al., 2001; Cho et al., 2001b). Furthermore, it was found that Akt1 knockout restricted the development of the placenta, leading to an impaired nutrient supply of the embryo. This may partially explain the reduced size of *Akt1*-/- knockout mice (Cho et al., 2001b; Yang et al., 2003). The phenotype of *Akt2*-/- knockout mice was more striking (Cho et al., 2001a; Garofalo et al., 2003). Consistent with the high expression levels of Akt2 in insulin-responsive organs and tissues, *Akt2*-/- knockout mice developed severe diabetes. Insulin action was blocked in the absence of Akt2 and the mice were insulin resistant, with hyperglycaemia, hyperinsulinaemia and glucose intolerance (Cho et al., 2001a). *Akt3*-/- mutant mice did not show a growth retardation syndrome. In adult *Akt3*-/- knockout mice, brain size and weight were dramatically reduced, which is caused, at least partially, by a significant reduction in both cell size and cell number (Tschopp et al., 2005), suggesting a critical role of Akt3 in the postnatal development of the mammalian brain. Finally, double knockout mice of Akt1/2 died shortly after birth, showing more obvious phenotypes than single knockout mice (Peng et al., 2003). The mutant mice were much smaller than wild type, with impaired skin development, skeletal muscle atrophy and abnormal bone development. Furthermore, the differentiation of double knockout mouse embryonic fibroblasts into adipocytes was blocked. This underscores the hypothesis that adipogenesis, the generation of new adipocytes, is under the control of Akt (Peng et al., 2003).

3.4.1.4 Domain structure of Akt

All three Akt isoforms consist of a conserved domain structure: an N-terminal PH domain, a central kinase domain and a C-terminal serine-threonine rich regulatory domain (RD) with a conserved hydrophobic motif (Fig. 3). The PH domain was originally found in pleckstrin, the major phosphorylation substrate for PKC in platelets (Tyers et al., 1988). The PH domain interacts with membrane lipid products such as phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), which is produced by class I PI3K. Biochemical analysis revealed that the PH domain of Akt binds to both, PIP_3 and phosphatidylinositol-(3,4)-bisphosphate (PIP_2), with similar affinity (Frech et al., 1997; James et al., 1996).

The kinase domain of Akt, located in the central region of the molecule, shares a high similarity with other AGC kinases such as PKA, PKC, p70 S6 kinase (p70S6K) and p90 ribosomal S6 kinase (p90RSK) (Peterson and Schreiber, 1999). A conserved threonine residue (T308 in Akt1), whose phosphorylation is required for enzymatic activation, is located in the activation loop of the kinase domain (Peterson and Schreiber, 1999). The RD domain is a C-terminal extension of around 40 amino acids, in which the C-terminal serine (S473 in Akt1) resides. The RD domain possesses the F-X-X-F/Y-S/T-Y/F hydrophobic motif (where X is any amino acid) that is characteristic of the AGC kinase family (Peterson and Schreiber, 1999). For all AGC family kinases, phosphorylation of the S or T residue in this hydrophobic motif is necessary for full activation of the kinase. Deletion of this motif completely abolishes enzymatic activity (Andjelkovic et al., 1997).

3.4.1.5 Activation of Akt

Akt is activated by a variety of stimuli, including growth factors such as platelet derived growth factor (PDGF), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor I (IGF-I), but also protein phosphatase inhibitors and cellular stress (Burgering and Coffer, 1995; Franke et al., 1995; Liu et al., 1998; Meier et al., 1997). Activation of Akt by these ligands is PI3K-dependent (Burgering and Coffer, 1995; Cross et al., 1995; Franke et al., 1995; Kohn et al., 1995) and blocked by the PI3K inhibitors wortmannin and LY294002, or by the dominant negative form of class I PI3K (reviewed in (Chan et al., 1999)). This class of PI3K forms heterodimeric enzymes consisting of a catalytic subunit and a

regulatory subunit. The regulatory subunit contains Src homology 2 (SH2) domains in their N- and C-terminal region to bind phosphotyrosine motifs of receptor tyrosine kinases and their substrates, such as insulin receptor substrate-1 (IRS-1). Thereby PI3K is targeted to the plasma membrane to access its substrates – the phosphoinositides, which are anchored in the plasma membrane.

The production of phosphoinositides by PI3K is reversed by phosphoinositide phosphatases. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) possesses 3'-phosphoinositide phosphatase activity, and activation of PTEN results in inactivation of Akt (Maehama and Dixon, 1999; Stambolic et al., 1998). Genetic alteration of the gene encoding PTEN has been found in nearly all types of human cancers examined so far (Li et al., 1997; Risinger et al., 1997; Steck et al., 1997; Tashiro et al., 1997), underscoring the role of PTEN as tumor suppressor, but also of Akt as protooncogene.

Phosphorylation site mapping of Akt from quiescent cells and IGF-I stimulated cells revealed a complex pattern (Alessi et al., 1996a). S124 and T450 in Akt1 are constitutively phosphorylated and seem to contribute to the stabilization of Akt protein. Phosphorylation of T308 and S473 in Akt1 is detected when cells are stimulated, and can be completely blocked by the PI3K inhibitor wortmannin (Alessi et al., 1996a). Point mutations at these sites with alanine (T308A and S473A for Akt1) show little activity, even after insulin or IGF-I stimulation, and the phosphorylation-mimicking mutant (T308D/S473D) shows constitutive kinase activation. There, these two sites are necessary and sufficient for full activation of Akt. The significance of the phosphorylation of these two sites was recently clarified by solving the crystal structure of the kinase (Auguin et al., 2004; Milburn et al., 2003; Thomas et al., 2002; Thomas et al., 2001).

The kinase responsible for T308 phosphorylation was purified by two groups (Alessi et al., 1997; Stephens et al., 1998) and identified as phosphoinositide-dependent kinase 1 (PDK1), a 63-kDa serine-threonine kinase. The primary structure of this kinase is similar to other AGC kinase family members, and it has a high-affinity PH domain at its C-terminal (Mora et al., 2004). For both proteins, Akt and PDK1, translocation to the plasma membrane for binding of PIP_3 is necessary for full activation (Anderson et al., 1998; Andjelkovic et al., 1997). The role of PDK1 for

T308 phosphorylation was confirmed by gene targeting in mouse embryonic stem (ES) cells. The deletion of PDK1 in ES cells resulted in inhibition of Akt, p70S6K, and p90RSK activation, with complete inhibition of phosphorylation on the threonine residue in their activation loops. Other AGC kinases were only partially inactivated in PDK1-/- cells (Williams et al., 2000), demonstrating the in vivo role and substrate specificity of PDK1.

The identity of S473 kinase is much more complicated and highly controversial. Since S473, as well as T308, phosphorylation is dependent on PI3K activity, PDK1 was initially assumed to be the kinase for S473 (Balendran et al., 1999). However, in *PDK1-/-* knockout ES cells S473 was phosphorylated similar to wild-type cells, whereas T308 phosphorylation was completely abolished (Williams et al., 2000). There are few reports describing Akt itself as a S473 kinase under certain conditions (Toker and Newton, 2000). However, as in PDK1-/- cells, T308 phosphorylation and Akt activity are completely abolished, but the S473 residue is still phosphorylated in response to IGF-I stimulation (Williams et al., 2000). Therefore, autophosphorylation is unlikely to play an important role for S473 phosphorylation.

Up to now ten kinases have been tested as Akt- S473 kinases (Dong and Liu, 2005). One candidate, mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) possesses the potential for Akt S473 phosphorylation (Alessi et al., 1996b), but its activation is not induced by Akt-activating stimuli, such as IGF-I, and is not dependent on PI3K activation, suggesting that this protein is not a major kinase for Akt S473 (Shaw et al., 1998). Integrin-linked kinase (ILK) 1, which possesses a phosphoinositide-binding motif, is activated by insulin in a PI3K-dependent manner. It can phosphorylate Akt on S473 in vitro (Delcommenne et al., 1998) and interacts with Akt in response to serum stimulation in vivo (Persad et al., 2001). However, as ILK1 kinase dead mutants can induce phosphorylation of S473 (Lynch et al., 1999) and fibroblasts from *ILK1-/-* knockout mice exhibit the same extent of Akt phosphorylation on S473 as wild-type mouse (Sakai et al., 2003), ILK1 is probably not important for S473 phosphorylation. Other proteins, which have been suggested to act as S473 kinase are, among others, PKCα, PKCβ, and mammalian target of rapamycin (mTOR) (reviewed in (Dong and Liu, 2005)) with mTOR being one of the most promising candidates for S473 phosphorylation so far (Hresko and Mueckler, 2005), at least in 3T3-L1 adipocytes. Therefore, it has been hypothesized that, unlike T308 phosphorylation, the S473 phosphorylation is mediated by more than one kinase

(Dong and Liu, 2005) and also might be cell type- and stimulus- specific (Fayard et al., 2005).

3.4.1.6 Cellular functions of Akt

After activation, Akt phosphorylates numerous proteins in both the cytoplasm and the nucleus. The minimum motif in a peptide enabling Akt phosphorylation is R-X-R-Sma-Sma-S/T-Hyd, where X is any amino acid, Sma is preferably a small residue other than glycine, and Hyd is a bulky hydrophobic residue, such as phenylalanine or leucine (Alessi et al., 1996b). For clarity, I will group the cellular functions of Akt in six categories: anti-apoptosis, cell cycle progression, protein synthesis, cellular proliferation, angiogenesis, and glycogen synthesis. A seventh category, the insulin-dependent glucose uptake will be dealt with in detail later.

3.4.1.6.1 Anti-apoptosis

In numerous cell types, it has been shown that Akt induces cell survival and suppresses apoptosis induced by various stimuli. One major target of Akt is the proapoptotic protein BAD, the Bcl-2/Bcl-XL-antagonist, causing cell death. By binding to Bcl-2 and Bcl-XL, BAD inhibits their anti-apoptotic potential (Datta et al., 1997). BAD phosphorylation by Akt inactivates BAD. It has also been shown that Akt activates p21 activated kinase 1 (PAK1), which in turn phosphorylates BAD resulting in its release from Bcl-XL complex (Datta et al., 2000; Tang et al., 2000). Akt also promotes anti-apoptosis by phosphorylation and inactivation of the protein Bcl-2-associated X protein (BAX) (Gardai et al., 2004), a promoter of cell death (Eskes et al., 1998; Granville et al., 1999; Murphy et al., 2000; Shinoura et al., 1999).
Akt furthermore inhibits apoptosis at the postmitochondrial level (Zhou et al., 2000) by phosphorylating the protein X-linked inhibitor of apoptosis protein (XIAP) (Dan et al., 2004). Activated XIAP prevents apoptosis by direct interaction and inhibition of activated caspases 9, 3, and 7 at postmitochondrial level (Deveraux et al., 1999; Deveraux et al., 1998; Roy et al., 1997).
Akt can also interfere with apoptosis by phosphorylation, and thus activation, of various cell survival related molecules. Phosphorylation of apoptosis signal-regulating kinase1 (ASK1) by Akt inhibits ASK1-dependent stimulation of c-Jun N-terminal kinase (JNK) (Kim et al., 2001; Yoon et al., 2002; Yuan et al., 2003), which plays an

essential role in anti-tumor reagents-induced programmed cell death (Benhar et al., 2001; Sanchez-Perez et al., 1998).

Furthermore, Akt phosphorylates the forkhead transcription factor Forkhead box, class O (FoxO) (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999), an important inducer of apoptosis (Lin et al., 1997). Akt phosphorylation of FoxO results in translocation of the transcription factor out of the nucleus and consequently its inactivation (Brunet et al., 1999; Kops et al., 1999). Similarly, Akt can phosphorylate the pro-apoptotic transcription factor Yes-associated protein (YAP). As in the case for FoxO, this results in the cytosolic localization and therefore inactivation of YAP (Basu et al., 2003).

3.4.1.6.2 Cell cycle progression, protein synthesis and cell differentiation

Akt targets several key cell cycle regulators including p21cip1/waf1 (Li et al., 2002; Rossig et al., 2001; Zhou et al., 2001a), p27kip1 (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002), and mouse double minute 2 (MDM2) (Ashcroft et al., 2002; Zhou et al., 2001b). In the case of p21cip1/waf1 and human p27kip1, which are both major inhibitors of cyclin-dependent cell cycle progression, phosphorylation by Akt causes the exclusion of these proteins from the nucleus.

An interesting target of Akt is the serine-threonine kinase mTOR, a regulator of cell cycle progression and cell proliferation by integrating signals from nutrients – such as amino acids and growth factors (Heitman et al., 1991; Helliwell et al., 1994; Kunz et al., 1993). mTOR regulates protein synthesis by initiation of mRNA translation leading to increased cell growth and subsequently increased proliferation (Hay and Sonenberg, 2004). Akt can directly phosphorylate mTOR (Bolster et al., 2003; Nave et al., 1999; Reynolds et al., 2002; Sekulic et al., 2000), but most likely acts on mTOR indirectly. Akt phosphorylation of the tumor suppressor protein tuberous sclerosis complex 2 (TSC2) leads to degradation of the TSC1/TCS2 complex and thus release of mTOR from TSC1/2-dependent inhibition (Dan et al., 2002; Hay and Sonenberg, 2004; Inoki et al., 2002; Manning et al., 2002; Plas and Thompson, 2003; Potter et al., 2002; Tee et al., 2003).

Furthermore, a crosstalk between Akt- and Raf- mediated signal transduction has been recently described in our laboratory (Moelling et al., 2002; Rommel et al., 1999;

Zimmermann and Moelling, 1999). This Akt- Raf1 crosstalk was found to regulate the decision whether a cell will differentiate or proliferate.

3.4.1.6.3 Angiogenesis

Accumulated evidence shows that Akt plays a central role in angiogenesis, the sprouting of new blood vessels. Akt mediates many angiogenic growth factors and regulates downstream target molecules involved in blood vessel growth, as recently demonstrated in *Akt1-/-* knockout mice (Chen et al., 2005). One of these target molecules is the vascular endothelial growth factor (VEGF), the primary inducer of angiogenesis, whose mRNA expression is induced by stabilization (Zhong et al., 2000) and enhanced translation (Laughner et al., 2001) through regulation of the mTOR pathway. Moreover, Akt phosphorylates and activates endothelial nitric oxide synthase (eNOS) (Dimmeler et al., 1999), which leads to production of NO and angiogenesis.

3.4.1.6.4 Glycogen synthesis

In humans, skeletal muscle store large parts of insulin-stimulated whole-body glucose uptake as glycogen (Shulman et al., 1990). Insulin promotes glycogen synthesis both by increasing rates of glucose transport (see below) and by activating glycogen synthase (GS), which catalyzes the final step in glycogen synthesis. Akt-mediated inactivation of glycogen synthase kinase-3β (GSK-3β) leads to reduced phosphorylation of GS, and therefore increased glycogen synthesis as demonstrated by overexpression of an Akt insensitive mutant of GSK-3β, which caused decreased insulin-stimulated GS activity (Summers et al., 1999a; Takata et al., 1999; Ueki et al., 1998). Nonetheless, the absolute contribution of Akt to the regulation of glycogen synthesis remains uncertain because several other signaling molecules also modulate this process.

3.4.2 Atypical PKC (aPKC)

3.4.2.1 Isoforms of the aPKC family

Protein kinase Cζ (PKCζ) was originally discovered as a unique PKC isotype (Ono et al., 1989). In the beginning of the 1990's, the PKCλ/ι isotype was cloned as the tenth member of the PKC family based on the amino acid sequence similarity of its kinase domain with those of other PKC members. Note that human PKCι (Selbie et al., 1993) and mouse PKCλ (Akimoto et al., 1994) are orthologs with 98% overall amino acid sequence identity and thus will be referred to as PKCλ/ι, hereafter. Comparison of the amino acid sequence supports the notion that PKCλ/ι is a close relative of PKCζ: the kinase domain of PKCλ/ι shows 86% identity with PKCζ and 45–55% with other PKCs (Akimoto et al., 1994). The cysteine-rich sequence of PKCλ/ι also displays higher amino acid sequence identity with PKCζ (69%) than with other PKCs (30–40%) (Akimoto et al., 1994). Based on these results, PKCλ/ι and PKCζ were categorized into a third subgroup of the PKC family, the atypical PKCs (aPKCs). The other two subgroups of the PKC family are the conventional PKC (cPKC) isoforms PKCα, PKCβI, its splice variant PKCβII (Coussens et al., 1987), and PKCγ, and the novel PKC (nPKC) isoforms PKCδ, PKCε, PKCη, and PKCϕ. Together they form the PKC family of S/T protein kinases (Ohno and Nishizuka, 2002). The cPKC isoforms have functional C1 domains, which bind phosphatidylserine and diacylglycerol (DAG) as well as phorbol esters. Furthermore, they possess a C2 domain, which binds anionic lipids and Ca^{2+}. Activation of cPKCs occurs by DAG in a Ca^{2+}- and phospholipid-dependent manner. Novel nPKC isoforms exhibit a functional C1 domain and a novel C2 domain, which does not bind Ca^{2+}. As a consequence, nPKCs are also activated by DAG and phospholipids, but are Ca^{2+}-insensitive. The atypical PKC isoforms only possess an atypical C1 and no C2 domain, and are insensitive to both DAG and Ca^{2+} (Mellor and Parker, 1998; Nishizuka, 1984).

3.4.2.2 Domain structure of aPKC

Proteins of the aPKC subfamily consist of four functional domains and motifs, including a Phox and Bem-1 (PB1) domain in the N-terminus, a pseudosubstrate (PS) sequence, a C1 domain and a kinase domain in the C-terminus. The structure of aPKC is depicted in Fig. 4.

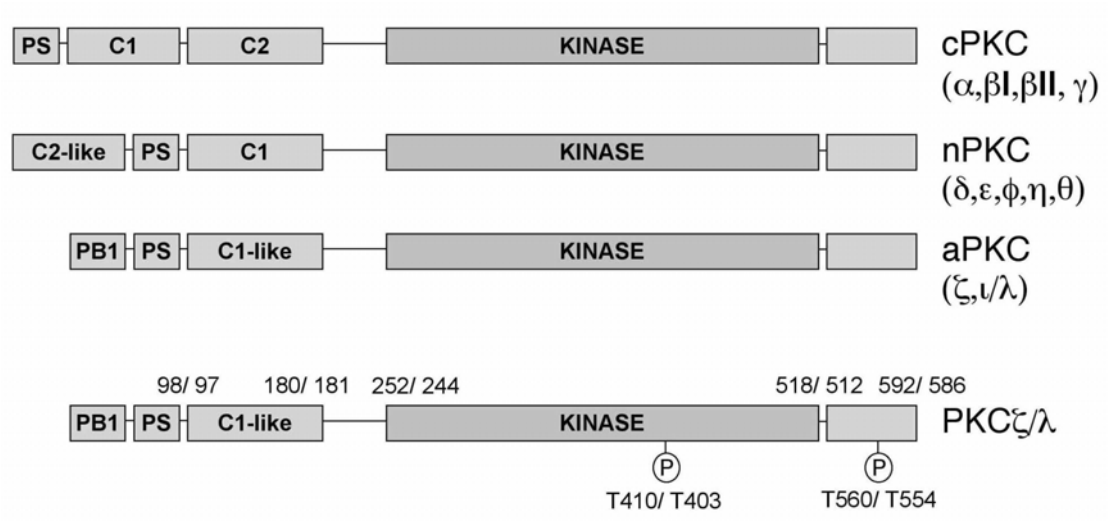

Fig. 4: At the top, the modular nature of the protein kinase C (PKC) isoforms is depicted. All PKC isoforms contain a conserved kinase domain (KINASE) that is autoinhibited by the pseudosubstrate (PS) domain, which blocks the active site in the unstimulated state.
The conventional isoforms (cPKC) contain a C1 domain to bind DAG or phorbol esters, and a C2 domain that binds the headgroup of phosphatidylserine in a calcium-dependent manner.
The novel PKC isoforms (nPKC) contain a functional C1 domain and a novel, nonfunctional C2-like domain, which does not contain the amino acids required for calcium-binding and thus renders nPKC calcium-independent.
The atypical PKC isoforms (aPKC) contain a PB1 domain for hetero- and homodimerization and a non-functional C1 domain, but do not contain a C2 domain and are thus DAG and calcium-independent.
At the bottom, the structure of PKCζ/λ, two members of the aPKC subfamily, is depicted. The essential threonine and serine residues necessary for activation are indicated. For further details see text.

The PB1 domain has been found, up to now, in more than 200 signaling molecules (Hirano et al., 2005) on the SMART database (Schultz et al., 1998). The PB1 domain of PKCι, which contains an OPR/PC/AID (OPCA) motif with conserved acidic and hydrophobic residues, has recently been crystallized (Hirano et al., 2005). It serves as binding motif to the OPCA motifs of a number of aPKC- interacting proteins, such as the proteins partitioning defective-6 (PAR-6), PKC zeta-interacting protein (ZIP), and mitogen activated protein kinase kinase 5 (MEK5) (Hirano et al., 2005; Ponting et al., 2002) through PB1- mediated heterodimerization or homo-oligomerization.

The PS domain is a short stretch of amino acids, which resembles the PKC substrate sequence except for A occupying the position of S or T as a phospho-group acceptor, and is assumed to block the substrate-binding cavity of the kinase domain as an autoinhibition mechanism (House and Kemp, 1987; Pears et al., 1990).

The C1 domain of aPKC is different in terms of a repeat structure from that of cPKC and nPKC that contain two repeated zinc-finger motifs, C1A and C1B, both of which are essential for interaction with and activation by the second messengers DAG and

phorbol ester. Although aPKC possesses a atypical C1 domain, it does not respond to DAG and phorbol esters (Ways et al., 1992). Thus, the function of the C1 zinc finger in aPKC is unkown (Mellor and Parker, 1998).

The kinase domain of aPKC, as well as other members of the AGC group, includes an ATP-binding region, an activation loop, a turn motif, and a hydrophobic motif. Recent analysis of the crystal structure of PKCι demonstrated that the conformation of active aPKC is strikingly similar to the kinase domain of Akt after activation (Messerschmidt et al., 2005). The ATP-binding region contains a lysine residue, which is crucial for its kinase activity. Important threonine residues for activation of aPKC are found in the activation loop (T410 of PKCζand T403 of PKCλ/ι, respectively) and in the turn motif (T560 of PKCζ, and T554/T555 of PKCλ/ι, respectively) and are phosphorylated upon activation (see below).

3.4.2.3 Activation of aPKC

Beside activation by PIP_3 (Nakanishi et al., 1993), which is essentially identical with activation of Akt described above aPKC can also be activated by other lipid components, such as phosphatidic acid (Limatola et al., 1994), arachidonic acid (Muller and Sorrell, 1995), and ceramide (Muller et al., 1995).

Unlike Akt, aPKC does not possess a PH domain, but the hydrophobic motifs of aPKC include a short sequence, F-E-G-F-E-Y, which is very similar to that of the PDK1-binding sites of PKC-related protein kinases, F-X-X-F-D-Y, where X is any amino acid (Parekh et al., 2000). In the activation loop of PKCζ, T410 is phosphorylated by PDK1 (Chou et al., 1998; Le Good et al., 1998). In ES cells lacking PDK1 as a result of genetic manipulation, PKCζ is not phosphorylated at T410, suggesting that PKCζ is a physiological substrate of PDK1 (Balendran et al., 2000b). While an alanine mutant of PKCζ, T410A, loses enzymatic activity, a glutamic acid–mutant, T410E, mimicking a phosphorylated threonine, retains its activity (Balendran et al., 2000b; Chou et al., 1998; Standaert et al., 1999), suggesting that T410 phosphorylation is essential for PKC activation.

Following the T410 phosphorylation, PKCζ presumably exposes the kinase domain for further phosphorylation. T560 in the turn motif of PKCζ is a key residue for

activation (Le Good et al., 1998). In PKCα and PKCßII, the phosphorylation of T638 and T641, respectively, corresponding to T560 in PKCζ is required for their catalytic functions and for locking these kinases in a catalytically competent state (Bornancin and Parker, 1996; Edwards et al., 1999). The T410E active mutant of PKCζ shows autophosphorylation in vitro, while T560A and T560E mutants do not, indicating that T560 is the only autophosphorylation site in PKCζ (Standaert et al., 2001). In living cells, whether T560 of PKCζ is phosphorylated by itself, by another PKCζ intermolecularly, or by other protein kinases including other PKC isotypes, remains to be resolved.

Numerous studies found that PKCζ and PKCλ/ι are activated and function interchangeably, at least in certain cellular processes such as the translocation of GLUT4 vesicles to the plasma membrane (Bandyopadhyay et al., 1999a; Standaert et al., 1999). For example kinase-inactive forms of both, PKCζ and PKCλ, inhibited translocation of the glucose transporter 4 comparably. Moreover, inhibitory effects of each of these kinase-inactive aPKC isoforms on the translocation process can be reversed by the wild type form of either atypical PKC, ζ or λ (Bandyopadhyay et al., 1999a).

3.4.2.4 Cellular functions of aPKC

A large number of studies have been performed in the last years to determine the physiological functions of aPKC. At present, most enzymatic and cell biological features revealed were found to be essentially common to PKCζ and PKCλ/ι. In many studies the high sequence identity between the aPKC isoforms hampered the investigation of the isoform specific effect of small interfering RNA (siRNA), dominant-negative mutants or pseudosubstrate peptides. Finally, all aPKC-binding proteins investigated so far, were reported to bind both isoforms. Therefore, I will restrict the following descriptions on the physiological role of atypical PKC isoforms on PKCζ, which has been better covered up to now.

3.4.2.4.1 Involvement of aPKC in the NF-κB pathway

In signaling for cell growth and survival, extracellular ligands often act through regulation of signaling pathways by transcription factors, such as the nuclear factor κB (NF-κB) (Luo et al., 2005). Kinase dead PKCζ blocks the response of NF-κB to these stimuli. This indicates that PKCζ is involved in NF-κB activation in signal transduction of tumor necrosis factor-α (TNF-α) and IL-1 (Sanz et al., 2000; Sanz et al., 1999). Furthermore, the PKC zeta-interacting protein ZIP and its homologs link aPKC to the TNF-α receptor signaling complex (Sanz et al., 1999) as well as to the IL-1- and the nerve growth factor (NGF)- receptor complexes (Sanz et al., 2000; Wooten et al., 2001a). ZIP is potentially also involved in the regulation of PKCζ activity (Chang et al., 2002b). PKCζ also regulates the NF-κB pathway by phosphorylating the ß-subunit of IκB kinase (IKK) (Lallena et al., 1999, Rahman, 2000 #1106; Savkovic et al., 2003), an essential component of the NF-κB pathway (Karin, 1999).

Collectively, aPKCs transduce signals from the receptors of TNF, IL-1, and NGF to the activation sites of NF-κB *in vivo*. Furthermore, they may be involved in a variety of signaling pathways from receptor complexes to the expression of their target genes by activation of transcription factors.

3.4.2.4.2 p70S6 kinase signaling cascade

aPKC directly associates with p70S6K in vivo, and dominant negative PKCζ suppresses the serum-induced activation of p70S6K (Akimoto et al., 1998; Romanelli et al., 1999), while constitutive active PKCζ synergistically enhances PDK1-induced phosphorylations and induces prolonged activation of p70S6K (Romanelli et al., 2002). Thus, aPKC apparently plays a role in p70S6K activation, although multiple signals, such as mTOR and Akt, are required for full p70S6K activation (Akimoto et al., 1998; Romanelli et al., 2002; Romanelli et al., 1999).

3.4.2.4.3 Cell polarity

Cell polarity is essential not only for cell functions but also for development and tissue maintenance. Recent studies have clearly demonstrated the importance of the

ternary complex of the proteins partitioning defective 3 homolog (PAR-3), PAR-6, and aPKC in cell polarity (Hurd et al., 2003; Joberty et al., 2000; Lin et al., 2000; Ohno, 2001; Plant et al., 2003). A PAR-3-PAR-6-PKCζ complex controls the formation of tight junctions in epithelial cells (Suzuki et al., 2001). Overexpression of dominant negative aPKC (Suzuki et al., 2001) or the N-terminal regulatory domain of PKCζ (Gao et al., 2002) interferes with the establishment of cell polarity. In addition to epithelial cells, PKCζ also controls polarity in different neuronal cells (Etienne-Manneville and Hall, 2001; Kuroda et al., 1999). Furthermore, aPKC is crucial for anterior-posterior polarity in *Caenorhabditis elegans* and the apical-basal polarity of epithelial cells and neuroblasts in *Drosophila melanogaster* (Cox et al., 2001; Drubin and Nelson, 1996).

3.4.3 Akt and aPKC- regulating proteins

Accessory proteins that bind kinases and thereby regulate their kinase activity or subcellular localization play a key role in many signal transduction cascades. In the case of Akt, several proteins have been reported to be interaction partners of the kinase (Brazil et al., 2002). Many of them are substrates for Akt and, in most cases do not affect Akt kinase activity. However, a number of proteins have been found to regulate Akt activity. These proteins include the carboxy-terminal modulator protein (CTMP) (Maira et al., 2001), which probably keeps Akt in an unphosphorylated and inactivated state by physical protein–protein interaction, heat-shock proteins, which bind Akt isoforms after stress treatment (Konishi et al., 1997), causing Akt activation and inhibition of apoptosis (Rane et al., 2003), and growth factor receptor-binding protein 10 (Grb10), which is assumed to recruit Akt to the plasma membrane in response to cellular activation (Jahn et al., 2002). Furthermore, the binding of keratin-10 to Akt leads to translocation of the kinase to the cytoskeleton and thus to inactivation of its kinase activity and inhibition of cell proliferation (Paramio et al., 2001), while binding of the protein T-cell leukemia 1 (Tcl1) to Akt (Laine et al., 2000; Pekarsky et al., 1999) has been speculated to facilitate activation of Akt (Sarbassov et al., 2005).

aPKC interacting proteins were mostly found to bind to the N-terminal PB1 domain and some of them have been implicated in the regulation of aPKC activity (Suzuki et al., 2003). The interaction partner PAR-6 links aPKC to small GTPases (Joberty et al., 2000; Lin et al., 2000; Noda et al., 2001; Qiu et al., 2000; Suzuki et al., 2001) and

enhances its kinase activity (Yamanaka et al., 2001) in a GTP-dependent manner, while Src binding to aPKC upon stimulation leads to Y256- phosphorylation (Seibenhener et al., 1999; Vandenplas et al., 2002; Wooten et al., 2001b) and nuclear import (White et al., 2002) of aPKC.

In summary, the serine-threonine kinases Akt and aPKC are involved in a large variety of signal transduction pathways. However, there is little data so far on signaling events where both kinases act additively or synergistically to modulate the signal transduction cascade.

Both proteins appear to be involved in p70S6K-activation and therefore cell growth by inducing protein synthesis, but the exact contribution of each individual kinase is far from resolved (see above). There have also been reports on the role of Akt and PKCζ in stress signaling via ceramides, but it appears that the two proteins have opposite effects in this cascade (Bourbon et al., 2002; Powell et al., 2003; Zhou et al., 1998).

The only system in which the two ProF-interacting proteins were clearly and unambiguously found to play key roles and additively act on signaling events is the insulin-dependent translocation of the glucose transporter GLUT4 to the plasma membrane of adipose and muscle cells. In the last part of the introduction I will discuss this system in detail with special emphasis on the role of Akt and aPKC.

3.5 Insulin-dependent GLUT4 translocation and glucose uptake

3.5.1 Structure and function of the glucose transporter 4, GLUT4

Glucose is the primary energy source used in the animal kingdom, and numerous transporters have evolved to move glucose and other sugars across lipid bilayers (Tetaud et al., 1997; Walmsley et al., 1998). In mammals, glucose is cleared from the bloodstream by facilitative glucose transporters (GLUTs), which comprise a family of highly conserved transmembrane domain-containing proteins.

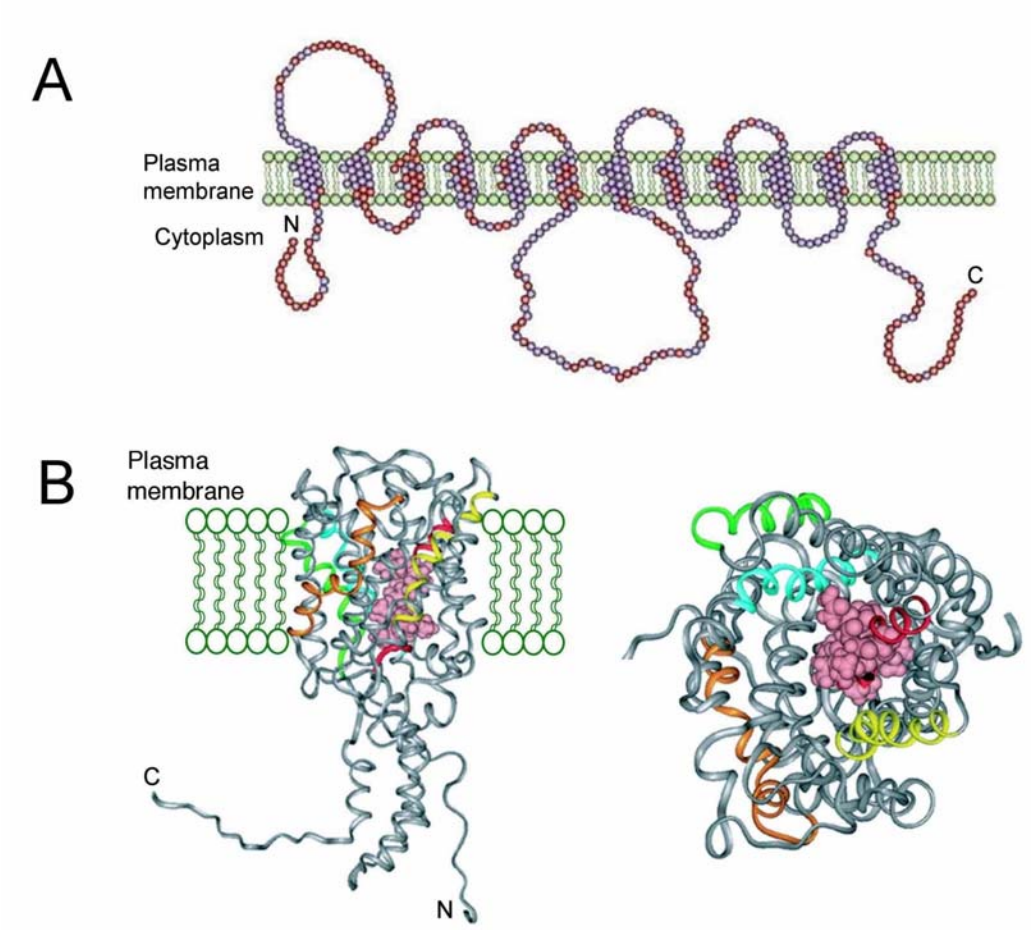

Fig. 5A: A schematic representation of the glucose transporter (GLUT) family of proteins is shown. They comprise 13 members at present, which are predicted to span the membrane twelve times with both N- and C-termini located in the cytosol. The diagram shows a homology plot between GLUT1 and GLUT4. Residues that are unique to GLUT4 are shown in red. For further details see text.

Fig. 5B: (Left) A ribbon representation (side view) of GLUT4 3D structure is depicted as model (Strobel et al., 2005). C- and N-terminal regions are shown as a coil due to lack of a suitable template. GLUT4 is depicted in a way that the extracellular or intravesicular region of GLUT4 is at the top. (Right) The extracellular or intravesicular region of GLUT4 is shown in top view in ribbon representation. Amino acids that are assumed to be important for glucose transport are shown in pink. The transmembrane helices of GLUT4 are colored: helix 2 is orange, helix 5 is yellow, helix 7 is red, helix 10 is green, and helix 11 is cyan. As can be seen, the transmembrane helices of GLUT4 fold into a a-helix barrel to form an aqueous pore for glucose transport across the plasma membrane.

The GLUT protein spans the membrane twelve- times with both, the N- and the C-terminus, located in the cytosol. It is assumed that this transmembrane structure forms an aqueous pore to transport glucose across the plasma membrane in an energy-independent manner (Fig. 5) (Joost and Thorens, 2001). Although the structural determination of GLUT membrane proteins by X-ray crystallography or nuclear magnetic resonance has been unsuccessful up to now (Alvarez et al., 1987), 3D-modeling approaches indicate that the glucose transporter isoform 4 (GLUT4) forms a right-handed α-helix barrel with twelve- transmembrane α- helix segments surrounding a central pore (Strobel et al., 2005), as can be seen in Fig. 5. Typically glucose transporters are localized in the plasma membrane. However, the glucose transporter isoform GLUT4, which is expressed primarily in adipose and striated muscle tissue, is unique among all GLUTs, by being sequestered in specialized intracellular membrane compartments under basal conditions and by being translocated to the plasma membrane after insulin stimulation (Bryant et al., 2002). Thereby, insulin stimulates the uptake of glucose into fat and muscle cells to promote the storage of sugar as intracellular triglycerides in fat and glycogen in muscle cells (Khan and Pessin, 2002).

3.5.2 The intracellular GLUT4 storage compartment

Under basal conditions, the majority of GLUT4 (more than 95%) is excluded from the plasma membrane of fat and muscle cells due to efficient endocytosis and intracellular sequestration (Holman and Cushman, 1994; Tanner and Lienhard, 1987). Insulin dramatically increases the exocytosis of GLUT4 (Bryant et al., 2002) and slightly inhibits its endocytosis (Czech and Buxton, 1993; Huang et al., 2001; Jhun et al., 1992; Yang and Holman, 1993), resulting in a rapid and strong increase in GLUT4 transporter levels at the plasma membrane. Interestingly, the increase in glucose transport activity due to insulin stimulation is approximately in the same range, indicating that GLUT4 translocation mediated most or all of the effect of insulin on this process (Thong et al., 2005; Zhou et al., 2004). There may also be to some degree a regulation of the GLUT4 catalytic activity (Harrison et al., 1992), but direct data on potential mechanisms involved are contradictory (Antonescu et al., 2005).

During the last decade considerable attention has been paid to the identification and characterization of the insulin-regulated GLUT4 storage compartment. The work of

several groups (Barr et al., 1997; Bogan and Lodish, 1999; Millar et al., 2000; Rea and James, 1997; Roh et al., 2001) led to the finding that GLUT4 is localized to a special membrane compartment that is separate from those occupied by other insulin-responsive secretory proteins, such as adipsin and leptin. This specialized insulin-responsive GLUT4 storage compartment will from now on be referred to as GLUT4 storage vesicles (GSVs).

The characterization of this GSV compartment has been very difficult, because at steady state GLUT4 is distributed throughout most of the endomembrane system (Kandror and Pilch, 1998). However, in the last years subcellular fractionation, vesicle immunoabsorption, membrane compartment ablation, and immunoelectron microscopy have revealed that around 50% of GLUT4 is localized to compartments of the general endocytic pathway. These compartments include endocytic markers, but are only weakly insulin-responsive and exclude the vesicle-associated membrane protein 2 (VAMP2), which will be discussed later (Hah et al., 2002; Martin et al., 1996; Ramm et al., 2000; Zeigerer et al., 2002). The rest of GLUT4 is found in the unique GSV compartment that is largely devoid of general endosomal markers (Livingstone et al., 1996; Robinson and James, 1992; Zorzano et al., 1989) and constitutively recycling proteins (Kupriyanova et al., 2002; Malide et al., 2000), but is highly insulin responsive (Birnbaum, 1992; Rea and James, 1997; Simpson et al., 2001). Electron microscopy revealed the GSVs as comparatively small (50 – 80 nm in diameter) tubulo-vesicular structures, which are distributed throughout the cytoplasm, rather than in typical endosomes (Malide et al., 2000; Slot et al., 1991).

3.5.3 Activation of GLUT4 translocation

The insulin-induced translocation of GLUT4 vesicles to the plasma membrane and the subsequent glucose entry is initiated by insulin binding to the insulin receptor, leading to activation of its tyrosine kinase activity and autophosphorylation (White and Kahn, 1994). Activated IR, in turn, phosphorylates tyrosyl residues in two subsets of phosphorylated substrates, involved in insulin action on GLUT4 vesicle translocation: the IRS proteins (White, 1998) and adaptor containing PH and SH2 domains (APS) proteins (Moodie et al., 1999; Ribon and Saltiel, 1997). The first subset of substrates initiates a signaling cascade that depends on class IA PI3Ks, whereas the second subset initiates a PI3K-independent signaling pathway. Activation of the PI3K-

dependent pathway is caused by tyrosine phosphorylation of IRS proteins that recruit and activate class IA PI3K through binding to the SH2 domain of the p85 regulatory subunit of PI3K. As discussed earlier, activation of the PI3K-dependent pathway increases cell surface production of PIP_3 (Yang et al., 2000), and perhaps PIP_2, which, in turn, recruits and activates downstream targets including serine-threonine kinase PDK1, and its substrate kinases Akt and PKCζ/λ.

The second pathway, which includes APS and the Rho family GTPase TC10 (Chiang et al., 2001), has been speculated to act in parallel with the PI3K-dependent pathway to allow GLUT4 translocation to the plasma membrane and glucose uptake membrane (Egawa et al., 2002; Frevert and Kahn, 1997; Guilherme and Czech, 1998; Isakoff et al., 1995; Jiang et al., 1998; Krook et al., 1997; Staubs et al., 1998), but has been questioned by recent siRNA studies (Mitra et al., 2004).

3.5.4 The role of Akt and PKCζ/λ in insulin-dependent glucose uptake

3.5.4.1 Akt

Insulin rapidly activates the serine-threonine kinase Akt (Funaki et al., 2004). Results from cell culture systems have generally supported the role of Akt in insulin-stimulated GLUT4 translocation (Ueki et al., 1998). Expression of constitutively active Akt increased glucose uptake and GLUT4 translocation to the plasma membrane, while dominant-negative Akt resulted in inhibition of glucose uptake (Czech and Corvera, 1999; Kohn et al., 1998; Kohn et al., 1996).

Among the Akt isoforms, Akt2 is assumed to preferentially mediate GLUT4 translocation, because Akt2 levels increase during differentiation of adipocytes (Hill et al., 1999; Summers et al., 1999b). Furthermore Akt2, but not Akt1, partially resides in GLUT4- positive membrane fractions (Calera et al., 1998) and has been recently shown to translocate to the plasma membrane upon insulin stimulation (Hanada et al., 2004), whereas Akt1 was reported to undergo nuclear localization after growth factor stimulation (Pekarsky et al., 2000). As mentioned earlier, knockout of Akt2, but not Akt1, resulted in mild glucose intolerance, impaired glucose uptake in skeletal muscle, and the failure of insulin to suppress hepatic glucose production (Bae et al., 2003; Cho et al., 2001a). These results, as well as recent studies with Akt gene

silencing in 3T3-L1 adipocytes (Jiang et al., 2003; Katome et al., 2003) strongly support the role for Akt2 in insulin-dependent glucose uptake.

3.5.4.2 aPKC

In addition to Akt, the aPKC isoforms PKCζ and PKCλ/ι have also been implicated in insulin-dependent GLUT4 translocation. Insulin stimulation results in an increase in PKCζ phosphorylation (Kanzaki et al., 2004; Kotani et al., 1998; Standaert et al., 2001) in a PI3K- (Bandyopadhyay et al., 1999b; Kotani et al., 1998; Sajan et al., 1999; Standaert et al., 2001; Standaert et al., 1999) and PDK1- (Chou et al., 1998; Le Good et al., 1998) dependent manner in adipocytes. Furthermore, insulin stimulation results in association of aPKC with GLUT4 vesicles (Braiman et al., 2001). Recruitment of aPKC to the plasma membrane upon insulin stimulation has been connected to its interaction with PDK1 (Balendran et al., 2000a; Le Good et al., 1998), but more recently large caveolin-positive rosette structures at the plasma membrane were assumed to be responsible for aPKC plasma membrane recruitment (Kanzaki et al., 2004).

Regardless of the exact mode of activation, translocation of aPKC to the plasma membrane and GLUT4 vesicles is well established (Braiman et al., 2001; Kanzaki et al., 2004; Standaert et al., 1999). Additionally, a large amount of data from cell culture experiments support a role for aPKC in insulin-regulated GLUT4 translocation. These include inhibition of aPKC activity with a pseudosubstrate peptide (Bandyopadhyay et al., 1997a) and general PKC inhibitors (Bandyopadhyay et al., 1997a; Bandyopadhyay et al., 1997b). In addition, expression of kinase-dead or inactive aPKC inhibited GLUT4 translocation and glucose transport, whereas expression of constitutively active aPKC promoted glucose transport (Bandyopadhyay et al., 2000; Kotani et al., 1998). Impaired activity of aPKC is also associated with insulin resistance and reduced glucose uptake in skeletal muscles of obese patients (Vollenweider et al., 2002) as well as impaired GLUT4 translocation in fat-fed rats (Tremblay et al., 2001). Only recently it has been shown that PKCλ knockout in ES cells and adipocytes impairs insulin-stimulated glucose transport (Bandyopadhyay et al., 2004), while earlier studies with siRNA-mediated gene silencing in 3T3-L1 adipocytes failed to show an effect of aPKC in glucose uptake (Zhou et al., 2004). Thus, it is important to explore further the discrepancies observed so far.

The connection between Akt2 and aPKC activation and GLUT4 translocation is still unknown. After insulin- stimulation active Akt2 and aPKC were found to rapidly bind to GLUT4-containing vesicles (Calera et al., 1998; Kupriyanova and Kandror, 1999; Standaert et al., 1999) and to the plasma membrane (Andjelkovic et al., 1997; Chen et al., 2003; Goransson et al., 1998; Standaert et al., 1999). However, the role of Akt2 and aPKC in the process of vesicle translocation is still largely unknown. Expression of kinase-inactive Akt1 (Ducluzeau et al., 2002) or Akt2 (Chen et al., 2003) fused to GLUT4 prevented GLUT4 translocation in response to insulin. This led to the assumption that activation of the kinases either at or in close proximity to GSVs in necessary for insulin to promote GLUT4 translocation (Chen et al., 2003; Ducluzeau et al., 2002; Welsh et al., 2005).

However, these data are not in accordance with a considerable number of translocation studies performed with inhibitors of PI3K and at low temperature (Bose et al., 2004; Elmendorf et al., 1999; Thurmond et al., 1998; van Dam et al., 2005). These results collectively suggest that Akt and PKCζ may play an important role at a late stage in the GLUT4 translocation to the cell surface, presumably involving fusion of GSVs to the plasma membrane. It is clear that more studies are necessary to fully unravel the role of those kinases. Therefore it has been assumed recently that the enzymes may impinge on more than one intracellular locus to exert their regulatory action on GLUT4 (Thong et al., 2005).

In summary, therefore, both kinases most likely phosphorylate a key substrate (or substrates) localized either close to the GLUT4 vesicles or the plasma membrane that plays a central role in translocation. Although several candidate substrate have emerged during the last years (Berwick et al., 2004; Hodgkinson et al., 2005a; Imamura et al., 2003; Kane et al., 2002; Sano et al., 2003; Yamada et al., 2005) our understanding of the kinase substrates and their role in GLUT4 translocation is yet far from complete. Given the importance of Akt2 and aPKC in the insulin-dependent GLUT4 translocation the detection of substrates of these kinases is of considerable interest.

3.5.5 Fusion of GLUT4 with the plasma membrane

Insulin stimulation leads to the rapid translocation of GSVs to the plasma membrane of muscle and adipose cells. A large number of results suggest an involvement of the

PI3K- targets Akt2 and PKC at this late step. In the presence of PI3K inhibitors GLUT4 vesicles accumulate in the vicinity of the plasma membrane (Bose et al., 2004) and PI3K-dependent regulation of SNARE proteins regulates GLUT4-vesicle docking and fusion to the plasma membrane (Min et al., 1999).

After translocation, the correct delivery of GLUT4-containing vesicles to the plasma membrane and the recycling of GLUT4 through intracellular membrane compartments relies, in part, on the recognition of target membranes by the vesicle and on correct couplings of proteins that direct the fusogenic process. This is performed by SNARE proteins - the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors - which play a critical role in a large variety of vesicular transport processes by regulating membrane docking and fusion (Rothman, 1994; Sollner et al., 1993; Sudhof, 2004; Weber et al., 1998). The transport vesicles contain proteins known as v-SNAREs, which form a class of coiled-coil proteins associated with the fusing membrane. The v-SNAREs bind in a highly specific manner to cognate membrane proteins, t-SNAREs, present in the appropriate target membrane. A defining feature of v- and t-SNAREs is the presence of a conserved α-helical SNARE domain. During SNARE-mediated membrane fusion, four of these SNARE domains contribute to a parallel four-helical bundle arrangement (Poirier et al., 1998; Sutton et al., 1998). In the case of the insulin-stimulated glucose transport in adipocytes syntaxin 4 and SNAP-23 have been implicated in GLUT4 vesicle trafficking as the required t-SNAREs (Foster and Klip, 2000; Watson et al., 2004).

Interestingly, the GLUT4 vesicles contain both v-SNAREs, VAMP2 and VAMP3, as found by indirect immunofluorescence microscopy (Malide et al., 1997; Martin et al., 1996). However, a number of studies indicate that VAMP2- and VAMP3-positive GLUT4 vesicles may reside in different subcellular compartments. They further indicate that VAMP2 is the v-SNARE located in the GSV compartment and responsible for insulin-stimulated GLUT4 translocation (Cheatham et al., 1996; Livingstone et al., 1996; Malide et al., 1997; Martin et al., 1998; Randhawa et al., 2000; Sevilla et al., 1997; Volchuk et al., 1994; Yang et al., 2001).

Furthermore, there is accumulating evidence that, in addition to v- and t-SNAREs, other proteins contribute to the membrane fusion process. Accessory molecules may bind to the SNARE proteins and may, upon insulin stimulation, modulate the SNARE-dependent fusion and docking events for GLUT4 surface externalization to

the plasma membrane (Grusovin and Macaulay, 2003). It has also been assumed, as described above, that the kinases Akt and aPKC may exert their main functions at this step, possibly either by phosphorylation accessory proteins or by acting on SNARE proteins by the aid of accessory proteins.

3.6 Aim of this thesis

Despite significant progress during the last years, many key questions concerning insulin-induced GLUT4 translocation still remain unsolved. Among the most exigent ones is the question on how insulin stimulation, via activation of Akt2 and aPKC, causes GLUT4 translocation to the plasma membrane and subsequent glucose uptake. Thus, the identification of kinase substrates of Akt2 and aPKC is of high importance and is ongoing at fast pace (Thong et al., 2005). However, these signal transduction cascades are likely to be very complex and probably tightly controlled by accessory or adaptor proteins to integrate kinases and substrates. Such adaptor molecules may play a significant role in GLUT4 translocation and glucose transport into adipocytes. More generally, Akt and aPKC are known to be involved in a numerous signaling events in a plethora of cellular systems. Therefore, the elucidation of signaling complexes that regulate activity, substrate phosphorylation or subcellular localization of these kinases would be of high relevance for a large variety of cellular systems.

The aim of my thesis is the characterization of one such potential adaptor protein. The WD-repeat propeller and FYVE-domain containing protein, designated as ProF, has recently been identified by members of our institute by the aid of a yeast two-hybrid screen using the kinase Akt as bait. The investigation of the role of Akt in cellular systems has been of great interest for our institute, and has led to the discovery of a crosstalk between Akt- and Raf- mediated signal transduction (Moelling et al., 2002; Rommel et al., 1999; Zimmermann and Moelling, 1999).

For this thesis, the interaction of ProF with Akt was studied, as well as the binding to the serine-threonine kinase PKCζ, both in overexpressed and endogenous systems. Since the binding of WD-repeat proteins to interacting kinases and other signal transduction-related proteins is often stimulation-dependent (Ron et al., 1994), special attention was paid to binding of ProF to Akt and aPKC upon cellular stimulation. The insulin-dependent translocation of GLUT4 vesicles in 3T3-L1 adipocytes was chosen as a model system to further investigate the biological role of ProF. This was done because of the key role of the ProF-interacting proteins Akt2 and PKCζ/λ in this pathway, the vesicular nature of ProF due to its FYVE domain, and the high expression levels of the protein in this cellular system. In adipocytes, the subcellular localization of ProF, Akt, and PKCζ was investigated, depending on insulin

stimulation. Overexpression or downregulation of ProF was performed in adipocytes to check its role in insulin-dependent signaling events such as glucose uptake. Knock down of ProF was also exploited to investigate the role of ProF in adipocyte differentiation. The influence of ProF in adipogenesis and lipogenesis was analyzed by various tools.

Furthermore, while this thesis was in progress, the v-SNARE protein VAMP2 was found as a novel interaction partner of ProF by members of our institute. The interaction of ProF with the v-SNARE VAMP2 and PKCζ was further investigated and the role of ProF as adaptor protein was analyzed in the PKCζ- VAMP2- complex. Additionally, the substrate phosphorylation of VAMP2 by PKCζ was investigated upon stimulation. Furthermore, it was tested whether ProF can influence the phosphorylation of VAMP2 in a PKCζ- and stimulation- dependent manner.

Although our knowledge on insulin-dependent GLUT4 translocation has tremendously advanced during the last years, substantial effort is still needed in order to understand the regulated trafficking of GLUT4. This accounts also for other vesicular proteins in adipocytes and in a wider variety of cellular processes. Adaptor proteins are likely to be of great importance in the adipocyte system (Kanzaki et al., 2004) – as well as in other pathways - and clearly deserve great attention. In that way, ProF might contribute as a potential adaptor protein to our understanding of signaling pathways in general, and insulin signaling, leading to glucose uptake in adipocytes, in particular.

4 Results

4.1 WD-FYVE protein binds the kinases Akt and PKCζ/λ and regulates glucose uptake in adipocytes
(submitted)

4.2 WD-FYVE protein binds VAMP2 and PKCζ and increases PKCζ-dependent phosphorylation of VAMP2
(submitted)

4.3 WD-FYVE protein regultes differentiation of preadipocytes and glucose uptake in adipocytes
(to be submitted)

4.1 WD-FYVE PROTEIN BINDS THE KINASES AKT AND PKCζ/λ AND REGULATES GLUCOSE UPTAKE IN ADIPOCYTES

(Manuscript submitted)

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Running Title: WD-FYVE protein regulates glucose uptake

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Summary

WD-repeat proteins offer a platform for protein-protein interactions by folding into a propeller- like structure. We identified the propeller-FYVE protein, ProF, consisting of seven WD-repeats and a FYVE domain for binding to membranes. ProF preferentially interacts with the activated protein kinases Akt/PKB and PKCζ/λ upon hormonal stimulation. Using adipocytes as a model system to study ProF function, we show that ProF translocates to the plasma membrane in response to insulin stimulation, parallel to the kinases Akt2 and PKCζ/λ and the glucose transporter type 4. Overexpression of ProF leads to increased glucose uptake upon insulin stimulation, while knockdown of ProF by small interfering RNA leads to reduced glucose uptake. Thus, we suggest that ProF functions as an interaction partner of kinases and regulates glucose transport into adipocytes. The protein may be involved in other inducible secretory systems.

Introduction

WD-repeat proteins belong to a large, structurally conserved protein family. Members of this family are characterised by the presence of several partially conserved sequence repeats of 40-60 amino acids. These repeats typically end with a Trp- Asp dipeptide (WD) at the C-terminus (1). The crystal structure of the ß-subunit of the GTP-binding protein transducin displays a highly

symmetrical propeller-like structure of seven folded WD-repeats (2) and all WD-repeat proteins are speculated to fold into such circularised ß-propellers. WD-repeat proteins serve as a platform for protein-protein interactions and are involved in a wide range of cellular functions such as signal transduction, RNA synthesis and processing, vesicular trafficking, and apoptosis (for reviews see (3)).

Well-characterised seven-bladed WD-repeat proteins involved in signaling are the receptors of activated C kinases (RACKs). These proteins bind activated protein kinase C (PKC) isoforms thereby enabling the interaction with their substrates (4).

Most WD-repeats are found in a multidomain context. Associated protein domains can specify the subcellular localisation of the molecule by binding to particular intracellular compartments. There the WD-repeats can bring together interacting partners at their correct site of action.

The FYVE domain, which was originally identified in Fab1p, YOTB, VAC1p, and EEA1 (5), represents one such compartment-specific targeting domain. FYVE domains are conserved from yeast to man. More than 30 FYVE domain-containing proteins are known in humans. The domain enables binding to phosphatidylinositol-3-phosphate (PI3P), which is mainly constitutively produced on endosomal membranes by phosphoinositide-3-kinase (PI3K). Most FYVE domain-containing proteins serve as regulators of endocytotic membrane trafficking, whereby a few proteins have a role in signaling or cytoskeleton remodelling (6). Their function is determined by additional domains such as coiled-coil domains in early endosomal antigen (EEA1), ankyrin repeats in Smad associated receptor antigen (SARA), or WD-repeats in the FYVE domain protein localised to endosomes-1 (FENS-1). While the function of FENS-1 is unknown, SARA presents mediators of the transforming growth factor (TGF)-ß signaling cascade to the TGF-ß receptor for phosphorylation (7) and EEA1 plays an important role in vesicle fusion during endocytosis by controlling endosome docking (8).

We identified ProF as interaction partner of the serine/threonine kinase Akt/PKB. Akt regulates multiple biological processes including survival, proliferation, cell cycle, and glycogen metabolism (9). We have recently described a crosstalk between Akt- and Raf- mediated signal transduction (10-12). Akt has three isoforms, Akt1, Akt2, and Akt3, each with overlapping but distinct cellular function. Akt1 plays an important role in growth and antiapoptosis, whereas Akt2 functions primarily as a regulator of glucose metabolism (13-16). Akt is activated by various growth factors and hormones such as insulin-like growth factor (IGF-1) or insulin. Activation occurs at the plasma membrane after PI3K-dependent generation of phosphatidylinositol-3,4,5-triphosphate (PIP_3). Thereby Akt is recruited to the plasma membrane via its pleckstrin homology (PH) domain and is activated by phosphorylation at Thr 308 by phosphoinositide-dependent kinase 1 (PDK1), followed by phosphorylation at Ser 473 by a yet unknown kinase. In addition to Akt, also atypical PKC isoforms, PKCζ/λ or its human orthologues PKCζ/ι, were found here to bind to the WD-FYVE protein. PDK1 also phosphorylates Thr 410/403 in the activation loop of PKCζ/λ in an insulin-dependent mechanism (17). PKCζ shows 86% overall amino acid sequence identity with PKCλ, but only 45 – 55% to other PKC isoforms (18). Insulin-mediated PKCζ/λ activation has been reported in a variety of cells, such as 3T3-L1 adipocytes (19). PKCζ/λ is involved in many signal transduction pathways, including insulin-dependent glucose uptake.

Both kinases have been shown to localise on vesicles and to translocate to the plasma membrane in response to insulin stimulation in striated muscles and adipose tissues (13,17,20). Insulin stimulates the uptake of glucose by inducing the translocation of vesicles containing the insulin-responsive membrane-spanning glucose transporter type 4 (GLUT4) from intracellular pools to the plasma membrane through a process of

targeted exocytosis (21). The steps involved in vesicle translocation are not fully resolved, but Akt and PKCζ/λ have been shown to play a key role in insulin-stimulated glucose uptake in 3T3-L1 adipocytes (22-24).

We describe here a new WD-repeat FYVE protein, designated ProF. It is targeted to internal vesicles and expressed in adipocytes and other tissues. It preferentially binds the activated kinases Akt and PKCζ/λ upon insulin stimulation. Furthermore, in differentiated adipocytes used here as a model system, ProF is translocated to the plasma membrane in response to insulin, similar to Akt, PKCζ/λ, and GLUT4. Overexpression or knockdown of ProF leads to increase or decrease of glucose uptake in response to insulin, respectively. Together, these data suggest a function of ProF in glucose transport into adipocytes.

Experimental procedures

ProF antiserum - An anti-ProF antiserum was raised in rabbits against a peptide corresponding to the 15 amino acids at the C-terminus of murine and human ProF. Some of the antiserum was thereafter affinity-purified to increase the specificity of binding (Eurogentech Belgium). The same peptide was used for competition.

Retroviral transduction and generation of stably transduced 3T3-L1 fibroblasts - Retroviruses containing the construct pRTP-Myc-ProF or the empty pRTP vector as control were produced using the BOSC-23 packaging cell-line as described (25,26). Early passage 3T3-L1 fibroblasts were incubated in virus-containing medium for 48 hours in the presence of tetracycline (40 ng/ml) to suppress ProF expression, yielding a pool of Myc-ProF expressing cells. The cells were used in the absence of tetracycline for subsequent glucose uptake and immunofluorescence studies.

For siRNA downregulation the sequence used was nucleotide (nt) 280 - 300 of ProF 3'-untranslated region (5'- CCA CTG TTA CCG CAA TCT A - 3') for siProF1, and nt 1154 – 1172 of ProF open reading frame, targeted against exon 11 (5'- GAA CTG ACA AGG TAA TTA A- 3') for siProF3. A 64-nt oligonucleotide, containing the target both in sense and antisense orientation was cloned into pSUPER and then together with the H1 promoter into the lentiviral vector FUGW (27). 4 x 10^5 HEK 293T cells were transfected with 0.6 µg of the lentiviral expression construct, 0.6 µg of HCMV-G and 0.6 µg pCMVDR8.3 helper virus plasmids using Lipofectamine 2000 (Invitrogen), followed by medium change after 24 h. The supernatant was used to infect 8 x 10^4 cells of low passage 3T3-L1 cells, yielding a pool of siProF- expressing cells.

Immunofluorescence - COS-7 or HeLa cells seeded on glass cover slips were transiently transfected with Fugene-6 or Lipofectamin. For inhibitor studies, cells were treated with 250 µM TPEN or 100 nM wortmannin (for 30 min). Cells were fixed with 3% paraformaldehyde and permeabilised in PBS containing 0.25% Triton X-100, incubated with appropriate primary and secondary antibodies and mounted in Mowiol (Hoechst Pharmaceuticals). Secondary antibodies were used at 1:100 dilution. The cells were examined by sequential excitation at 488 nm (FITC) and 568 nm (TRITC) using a confocal laser microscope (Leica SP2) and a 40x1.25 oil immersion objective (Leica). The images were processed using IMARIS (Bitplane).

Fully differentiated 3T3-L1 adipocytes were mildly trypsinised, replated on glass cover slips, serum-starved for 2 hours and stimulated with 100 nM insulin for the indicated time. The cells were fixed with 4% paraformaldehyde and permeabilised in PBS containing 1% Triton X-100 before incubation with appropriate first and secondary antibodies.

Immunoprecipitation and Western blot analysis - HEK 293T or HEK 293 cells were transfected with expression vectors encoding the indicated proteins and processed as described (10). IGF-1 stimulation was performed with 100 ng/ml IGF-1 for 15 min. Murine tissues were extracted in a glycerol buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.2% Nonidet NP40, 10% glycerol, 1 mM EDTA using a tight-fitting tissue blender. Complete homogenisation was achieved after 6-10 strokes, 10 seconds each.

Lysates were solubilised by shaking for 30 minutes at 4 °C.

Lysates of HEK 293T cells were immunoprecipitated with 1 µg of the appropriate antibody for at least 2 hours at 4 °C followed by additional 45 minutes with 10 µl of protein G-sepharose. Lysates and the resulting immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis as described (10).

Mouse brain lysates (approximately 1.25 x 10^6 cells/ml lysate) were subjected to immunoprecipitation with 1 µg of affinity-purified antibody and incubated overnight at 4°C with or without a 100-fold molar excess of the competing peptide. The endogenous ProF protein was detected as 44 kDa form in direct lysates and precipitates. A 40 kDa protein, also competed by the peptide, was observed in some cases with cultured cells and is likely to be unspecific.

In 3T3-L1 cells, immunoprecipitation of endogenous ProF was performed with anti-ProF-antiserum, covalently coupled to protein G-sepharose in order to avoid overlapping of predominant signal of the IgG antibody heavy chain. In brief, 10 µl of protein G-sepharose (Amersham) were incubated with 2 µg of anti-ProF antiserum in 500 µl of Washing-Binding Buffer (all buffers from Pierce) for 30 minutes at room temperature, afterwards washed twice with this buffer and incubated in 260 µl of Crosslinking Buffer with 850 µl of freshly added dimethylpimelidate. Thereafter protein G-sepharose was incubated for 1 hour at room temperature and washed twice with Crosslinking Buffer, incubated for 10 minutes with Blocking Buffer at room temperature, washed twice with Blocking Buffer, washed three times with Elution Buffer, and then equilibrated with Washing- Binding Buffer. Lysate of 3T3-L1 cells was added to beads and incubated overnight at 4°C.

Glucose uptake measurements - For experiment 6A, differentiated adipocytes were starved for 20 hours in DMEM containing 0.5% FCS, and stimulated for 1 hour with insulin in the presence of 1ml of 0.4 µCi/ml D-(U-^{14}C)-glucose (Amersham) with a specific activity of 311 mCi/mmol. Cells were lysed in radioimmunoprecipitation assay buffer (12) and lysates were subjected to scintillation counting analysis for measurement of ^{14}C-glucose uptake.

For experiment 6B, differentiated adipocytes were starved for 20 hours in DMEM containing 0.5% FCS, and glucose uptake analysis was performed as described in (28) with slight modifications. Briefly, cells were incubated for 30 minutes at 37°C with 1 mL of DMEM containing 100 nM insulin, washed three-times with 2 ml of warm HEPES-buffered Krebs- Ringer phosphate buffer (120 mM NaCl, 5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES, pH 7.2) and incubated for 10 minutes in the same buffer in the presence of 1ml of 1 µCi/ml 2-Deoxy-D-(1-^{3}H)-glucose (Amersham) with a specific activity of 0.8 Ci/mmol. Cell were lysed in radioimmunoprecipitation assay buffer (12) and lysates were subjected to scintillation counting analysis for measurement of ^{3}H-glucose uptake.

Reagents and antibodies - Cell culture media, supplements and Novex® 8 – 16% and 10–20% Tris- Glycine gradient gels were purchased from Invitrogen. N,N,N',N'-*tetrakis*(2-pyridylmethyl)ethylenediamine (TPEN), PKA inhibitor, and IGF-1 were obtained from Calbiochem and insulin was obtained from Novo Nordisk. Wortmannin, isobutylmethylxanthin (IBMX), dexamethason, and TRI Reagent were obtained from Sigma. Restriction enzymes were purchased from New England Biolabs and polymerases from Promega. The Access RT-PCR system was purchased from Promega, and the QuikChange™ Site-Directed Mutagenesis kit from Stratagene. Unlabelled ATP for in vitro kinase assay was obtained from Roche. Oligonucleotides were purchased from Microsynth (Balgach, Switzerland). The transfection reagents Lipofectamin and Fugene 6 were purchased from Invitrogen and Roche, respectively. Protease inhibitors were from Roche, protein G sepharose beads, Redivue [γ-^{32}P] ATP, 2-Deoxy- D-(1-^{3}H)-glucose, and D-(U-^{14}C)-glucose were obtained from Amersham Pharmacia Biotech. Mouse monoclonal antibody 9E10 against Myc and rat

monoclonal 3F10 against HA were obtained from Roche, rabbit polyclonal A14 against Myc, rabbit polyclonal Y-11 against HA, mouse monoclonal DF1513 against CD71/Transferrin receptor, mouse monoclonal F7 against human Akt2, goat polyclonal C-20 against GLUT4, rabbit polyclonal to PKC-isoforms α/ß, γ, ε, η, and ζ/λ, and a goat polyclonal against PKCζ/λ were obtained from Santa Cruz Biotechnology, mouse monoclonal 262K against HA, rabbit polyclonal against Akt, mouse monoclonal 5G3 against Akt, rabbit polyclonal against phospho-Akt-Thr308 and rabbit polyclonal against phospho-Akt-Ser473 from Cell Signaling Technology, sheep polyclonal against Akt1, sheep polyclonal against Akt2 and mouse monoclonal GD11 against c-Src and PKCsubstrate peptide from Upstate Biotechnologies, mouse monoclonal clone 14 against EEA1 from BD Transduction Laboratories, the anti-rabbit IgG phycoerythrin (PE) conjugate from Sigma and the mouse monoclonal 1F8 against GLUT4 was from Biogenesis. Fluorescein (FITC)-, rhodamine (TRITC)- and Cy™5-conjugated anti-donkey antibodies against mouse, rabbit or sheep were purchased from Jackson Biologicals.

Cell culture - The human embryonic kidney cell lines HEK 293 (ATCC number CRL-1573), HEK 293T (ATCC number CRL-11268), the human epithelial cell line HeLa (ATCC number CCL-2), the simian kidney cell line COS-7 (ATCC number CRL-1651) and the murine fibroblastic cell line 3T3-L1 (ATCC number CL-173) were grown in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal calf serum (FCS; Seratec). Penicillin and Streptomycin were added to cultures of 3T3-L1 cells.

Yeast two-hybrid screen - A yeast two-hybrid screen was performed as described (29) using full-length Akt1 as bait. A B cell-specific cDNA library was obtained from S.J. Elledge (Baylor College of Medicine, Houston, Texas) (30). One of two cDNAs identified in the yeast two-hybrid screen was subcloned into pBluescriptKS (Stratagene).

Computational analysis of ProF protein - Analysis of secondary structure elements were performed using the Simple Modular Architecture Research Tool (SMART; (31)) and a WD motif program (http://BMERC-www-bu.edu/wdrepeat). The three-dimensional model of ProFΔFYVE was generated by 3D-PSSM program (32) with the WD-repeat protein Tup1 (PDB 1ERJ) as structural template (33). The calculated fold-structure had a certainty of fit better than 95%.

Recombinant DNA procedures - A ProF fragment containing a double *myc*-tag 5'-prime to the cDNA was generated by PCR and the amplified fragment was cloned into pCMV5 generating pCMV5-myc-ProF.

The deletion mutant lacking the FYVE domain, ProFΔFYVE (pCMV5-myc-ProFΔFYVE) was generated by introducing a *Bsp*M1 restriction site between the FYVE domain and the last WD repeat domain by site-directed mutagenesis using pCMV5-myc-ProF as a template and the following primers: 5'-GGC CAT CAC AGA TGA AGA ACC TGC ACC CAC AGC CAC CTT CC-3' and the complementary reverse primer. Subsequently the plasmid was digested with *Mun*I and *Bsp*M1 restriction enzymes to excise the FYVE domain. The plasmid was annealed with a short partially overlapping oligonucleotide linker (forward primer: 5'-AAT TGA TCT CCT GTG GCG GTG ATG GTG GGA TTG TCG TCG GGA ACA TGG ACG TGG AGG AAC GTG CAC CC-3' and reverse primer 5'-CTG TGG GTG CAC GTT CCT CCA CGT CCA TGT TCC AGA CGA CAA TCC CAC CAT CAC CGC CAC AGG AGAT C-3') and religated. The ProF mutants lacking blades 1 to 3 and blades 4 to 7 were generated by site directed mutagenesis using pCMV5-myc-ProF or pCMV5-myc-ProFΔFYVE as templates generating pCMV5-myc-ProFΔFYVEΔ4-7(primers: 5'-GCA ATT TGC CTG GCA CTA GTC TGA GAG TGG GCA GC-3'and reverse complementary oligo) and pCMV5-myc-ProFΔ1-3 (primers: 5'-CGA ACA AAA ACT TAT TTC TGA AGA AGA TCT GCT ATG CTC TGA GAG TGG GCA GCG CCT GGG AGG-3') and -pCMV5-myc-ProFΔFYVEΔ1-3, respectively. A translational green fluorescent protein (GFP)-ProF fusion was generated by cloning myc-ProF into the *Bgl*II and *Sal*I digested plasmid pEGFP-C2 (Clontech). The

retroviral vector pRTP-myc-ProF was constructed by inserting the PCR amplified fragment into the unique *Eco*RI site of pRTP under control of a tetracycline repressible promoter (25). HA-Bcr wildtype was cloned in pCDNA3 (Invitrogen). The sequence of all plasmids was verified by DNA sequencing.

Haemagglutinin tagged (HA)-Akt1 wild-type (34), HA-Akt2 wild-type (35) and HA-m/p-Akt1 (36) have been described previously. c-Src wild-type cloned in pUSE plasmid was purchased from Upstate Biotechnologies. HA-PKCζ encoding plasmid was obtained from the Fraunhofer Institute, Stuttgart, Germany.

RNA extraction, Reverse Transcription-PCR (RT-PCR) and Real Time PCR - RNA was extracted using TRI Reagent and RT-PCR was performed with 1 µg of total RNA. Primers for human and murine ProF were: forward (nt 658-678): GAT CAC TCT GTC ATC ATG TGG; reverse (nt 894–915): CTT ACT GTC CCA CAT TTG CTT G. Primers for murine ß-actin as internal control were designed as described (37), primers for human ß-actin were: Forward (nt 607-629): ACG GCC GAG CGG GAA ATC GTG CG, reverse (nt 987-1009): ACT TGC GCT CAG GAG GAG CAA TG. Primers for murine GLUT4 were: Forward (nt 1220-1240): TTC ATT GTG GCA GAG CTC TTC, reverse (nt 1352-1372): GAC GGC AAA TAG AAG GAA GAC. First-strand cDNA was synthesized at 48 ˚C for 45 min, 2 minutes at 94 ˚C and 22 cycles (for ß-actin) or 30 cycles (for ProF) for 30 seconds at 94 ˚C, 1 minutes at 68 ˚C, and 2 minutes at 68 ˚C.

In vitro kinase assay - Myc-ProFΔFYVE protein was expressed in HEK 293T cells and immunoprecipitated with 1 µg of antibody as previously described. Beads were washed three times with ice-cold NETN buffer and twice with kinase buffer containing 200 mM Tris-HCl, pH 7.5, 200 mM $MgCl_2$ and 10 mM DTT, then resuspended in 40 µl of kinase buffer, including 3 µM PKA inhibitor, 20 µM unlabelled ATP, 10 mCi/ml [γ-^{32}P] ATP with a specific activity of 3000 Ci/mmol, and 0,45 µg PKC substrate peptide (Upstate Biotechnologies). G-beads were incubated at 30°C for 30 minutes and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Autoradiography of phosphorylated PKC substrate peptide was performed using a STORM PhosphorImager (Amersham Biotech) and evaluated with the STORM Software.

Differentiation of adipocytes - For differentiation, early passage 3T3-L1 fibroblasts, were grown in growth medium (DMEM supplemented with 10% FCS) to confluency followed by medium change. 72 hours later differentiation was induced by growth medium supplemented with 166 nM insulin, 0.1 µg/ml dexamethason, and 112 µg/ml 3-isobutyl-1-methylxanthine (IBMX). Daily replacement of the hormonal differentiation medium was performed for 3 days. Then, at day 0, the medium was replaced by growth medium containing 166 nM insulin. For insulin-stimulation experiments of pRTP-Myc-ProF transduced cells, the cells were starved in DMEM containing 0.5% FCS for 16 hours and stimulated for 1 hours as indicated in Figh. 6A. Cells were used for experiments within 20 days after removal of the hormonal differentiation medium. Differentiation was monitored by visual inspection or Oil Red O staining.

Subcellular fractionation - Adipocytes were stimulated with 100 nM insulin, homogenized with eight passages through a 21 $1^1/_2$ gauge needle (Becton Dickinson Microlance-3) and fractionated as described (38). The plasma membrane (PM) pellet was resuspended in 400µl buffer and purified on a 1.12 M sucrose cushion by centrifugation at 70,000 x g for 10 minutes. The PM-enriched interface (~ 500 µl) was thereafter diluted in homogenization buffer without sucrose and pelleted at 200,000 x g for 4 minutes. The PM and the low-density microsomal fraction (LDM) were resuspended in NETN buffer described above and incubated for 1 hour at 4°C to solubilise proteins, followed by centrifugation at 13,000 x g and 4°C for 10 minutes.

Results

We performed a yeast two-hybrid screen using a human B cell-specific cDNA library and full-length Akt1 as bait (29). One

of the identified clones encoded a novel protein of 400 amino acids and a predicted molecular mass of 44 kDa (accession number: AAL04162), provisionally designated as WD-FYVE protein 2 (WDFY2), (Fig. 1A). Analysis of the secondary structure using the specific WD motif search program BMERC, (http://BMERC-www-bu.edu/wdrepeat), identified seven WD-repeats. Database mining using the Basic Local Alignment Search Tool, BLAST, program (39) revealed a murine homologue of unknown function (accession number: NP-780755) with only 8 divergent amino acids (Fig. 1A). Fig. 1B shows alignments of some proteins with identical domain structure. Homologues of the protein were encountered in *Drosophila melanogaster* and *Caenorhabditis elegans*, indicating a conserved function of the protein in animals. No homologues of the protein were found in other eukaryotes or prokaryotes.

The individual WD-repeats of human ProF are shown in Fig. 1C with the aberrations compared to the consensus sequence (1) highlighted in grey and their number indicated in brackets. Analysis of the secondary structure suggested that all seven repeats are able to form four antiparallel ß-strands, A through D, indicated as arrows on the top of Fig. 1C. Seven WD-repeats can fold into a highly symmetrical ß-propeller (2), whereby one propeller blade contains the last three strands of a repeat unit followed by the first strand of the next repeat unit. Therefore, we used a protein structure prediction approach to find out whether ProF is able to fold into a seven blade-containing propeller (Fig. 1D). As template, we used the WD-repeat containing protein Tup1, a corepressor of transcription in yeast (33). Indeed, the amino acid sequence of the protein without the FYVE domain could be modeled into a seven blade-containing propeller (Fig. 1D) arranged around a central axis with a certainty of fit better than 95%. Thus, the propeller-FYVE protein was designated as ProF.

The FYVE domain of ProF is located between WD-repeats 6 and 7 and comprises two zinc ions coordinated by two times four conserved cysteine residues to form zinc fingers (6). To compare the FYVE domain of ProF with that of other proteins, we aligned the amino acid sequence of the FYVE domains of ProF, the human ProF-homologue FENS-1, and EEA1 in Fig. 1E. The predicted secondary structure of the EEA-1 FYVE domain is shown at the top. It contains four ß-strands and two a-helices, as indicated by arrows and boxes (Fig. 1E). The FYVE domains of ProF and FENS-1 are highly conserved, with 80% amino acid sequence identity, but both share only limited identities with the FYVE domain of EEA1, 27% to 28%, respectively. ProF and FENS-1 share a change of an R to Q, R-Q-H-H-C-R in the conserved R-(R/K)-H-H-C-R motif and an 11 amino acid insert between a_2 and $ß_1$ (Fig. 1 E, highlighted in yellow). These two characteristics are unique among all FYVE domain proteins (40,41). They may account for a higher affinity towards PI3P and may make the binding of a second protein, usually important for membrane targeting (42), obsolete.

To detect mRNA expression of endogenous ProF we performed reverse-transcription polymerase chain reaction (RT-PCR) using primers that recognise both the murine as well as the human ProF (m/h ProF). Endogenous ProF mRNA was detectable in murine brain extract, 3T3-L1 adipocytes, NIH-3T3 cells, and in human embryonic kidney HEK 293T cells, but undetectable in HeLa cells (Fig. 2A). We raised an antiserum against a peptide corresponding to the 15 C-terminal amino acids of mouse and human ProF. The antibody recognised a band at 44 kDa height, corresponding to ProF as demonstrated with the detection of overexpressed untagged ProF, (Fig. 2B, lane 4) and the downregulation of the band by a siRNA targeted against ProF (Fig. 2B, lane 2). We tested a number of additional cell lines and found the highest expression levels of ProF protein in 3T3-L1 cells (unpublished data). In a number of cell lines an additional band was detected at 40 kDa height (Fig. 2B, lanes 1 –3), which was not related to ProF as demonstrated by siRNA (Fig. 2B, lane 2).

Because FYVE domains bind to PI3P present on vesicles, we investigated the

subcellular localisation of ProF. For that purpose, we transiently transfected HeLa cells, which are devoid of endogenous ProF, with a Myc-tagged ProF construct and performed confocal immunofluorescence microscopy. Myc-ProF partially colocalised with EEA1, a marker for early endosomes, and to a lesser extent with the transferrin receptor (TfR), a marker for recycling endosomes (Fig. 2C). To analyse the role of the FYVE domain we compared the localisation of Myc-ProF and Myc-ProFΔFYVE lacking the FYVE domain in COS-7 cells. The vesicular localisation was dependent on the FYVE domain (Fig. 2D). Myc-ProFΔFYVE was distributed in the cytoplasm and the punctuated staining was strongly reduced in comparison to the full-length protein. Furthermore, the vesicular localisation of Myc-ProF was lost in the presence of the Zn^{2+}-chelator TPEN, which destroys zinc finger configurations. Additionally, the vesicular staining of a GFP-ProF fusion protein was abolished in the presence of wortmannin (Fig. 2D). The PI3K-inhibitor wortmannin prevented the formation of PI3P and PIP_3, which is required for the binding of FYVE domains to vesicles. These findings indicate that the FYVE domain indeed targets the protein to PI3P-containing vesicles.

The ability to dimerise is a property of a number of FYVE domain proteins and important for their affinity to endosomal membranes. Dimerisation of EEA1 is assumed to enhance the avidity to PI3P. Homodimerisation also takes place in the FYVE domain-containing protein SARA (43) and this plays an important role for its endosomal localisation. To demonstrate the oligomerisation of ProF we performed coimmunoprecipitation assays with Myc- and Flag-tagged ProF (Fig. 2E). Indeed, the interaction was clearly detectable. Since the dimerisation motif of EEA1 and SARA is localised in the FYVE domain (41) we tested the interaction of the ProF mutant ProFΔFYVE. The interaction also took place when only one of the interaction partners contained a FYVE domain. This suggests that sequences outside of the FYVE domain contribute to the interaction (Fig. 2E). This had consequences for our effort to design dominant-negative mutants of ProF. Putative dominant-negative ProF mutants deleted for the FYVE domain can still heterodimerise with the wild-type and can therefore behave similarly to the wild-type protein.

ProF was identified as an interaction partner of Akt1, which we wanted to confirm by coimmunoprecipitation assays. We tested the binding to other kinases, known to interact with WD-repeat proteins such as members of the PKC family (4) and the Src kinase family (44). Additionally, the multidomain protein break point cluster region, (Bcr) was tested, since it contains a PH domain and various PH domain-containing proteins are known to bind to WD-repeat proteins (45). We tested the kinases HA-Akt1, HA-PKCζ, and HA-Bcr as well as untagged Src by using transiently transfected HEK 293T cells and antibodies against the HA-tag or Src (Fig. 3A). Myc-ProF coimmunoprecipitated HA-Akt1 and HA-PKCζ, but not HA-Bcr or untagged Src. HA-Akt1 and HA-Akt2 interacted equally well with ProF (unpublished data). Furthermore overexpressed ProF coprecipitated endogenous atypical PKC isoforms PKCζ/λ in HEK 293T cells, but not the classical PKC isoforms PKCα/ß or the novel PKC isoform PKCε (unpublished data). These results suggest a specificity of ProF for particular kinases and even isoforms within a kinase family.

To further characterise the interaction of Akt and PKCζ with the ProF protein we mapped their interaction sites. Various mutants of ProF were constructed. However, deletion of single amino acids, fragments, or individual blades did not show reduced binding (unpublished data). Two other mutants comprised the blades 1 to 3 of ProF (Myc-ProFΔ4-7ΔFYVE), and blades 4 to 7 of ProF (Myc-ProFΔ1-3ΔFYVE). Furthermore, in both of these mutants the FYVE domain was deleted. They were tested by cotransfection with HA-Akt and HA-PKCζ (Fig. 3B). Akt was coprecipitated by both mutants, but bound more strongly to blades 1 to 3 (mutant Myc-ProFΔ4-7ΔFYVE), whereby PKCζ predominantly bound to blades 1 to 3. Akt truncation mutants containing either the PH

domain or the kinase and regulatory domains were found to still bind Myc-ProF (unpublished data). These results suggest that several binding sites contribute to the interaction between the kinases and ProF. Nevertheless, they indicate a specific binding of Akt1, Akt2, and PKCζ to ProF involving mainly blades 1 to 3.

The binding of kinases to WD-repeat proteins, involved in signal transduction, is often regulated through phosphorylation of the binding kinases (45). Therefore, HEK 293T cells were stimulated by IGF-1. Immunoprecipitates of overexpressed Myc-ProF from stimulated cells indeed contained both, more total Akt and more phosphorylated Akt in comparison to non-stimulated cells (Fig. 4A). Even in unstimulated cells, which contained only a low amount of activated Akt, phosphorylated Akt was preferentially recruited to ProF, indicating a very efficient selection. Likewise, immunoprecipitates of overexpressed Myc-ProF contained more PKCζ/ι, the human orthologue of murine PKCζ/λ, in stimulated cells (Fig. 4A). The coimmunoprecipitated PKCζ/ι is catalytically active, since it was able to phosphorylate a PKC substrate peptide in an in vitro kinase assay. These results suggest a stronger interaction of ProF with the active kinases and confirm the interaction of ProF with both kinases in a semi-endogenous system.

Endogenous ProF also interacted with endogenous Akt and PKCζ/λ, as shown by coimmunoprecipitation of the kinase- adaptor complexes in mouse brain lysates (Fig. 4B). Furthermore, the interaction of endogenous ProF with endogenous PKCζ/λ is shown by coimmunoprecipitation of PKCζ/λ in 3T3-L1 cells (Fig. 4C). A 40 kDa protein recognised by the anti-ProF antiserum in the direct lysate (Fig. 4C, lane 2) is of unknown origin. Thus, ProF interacts with PKCζ/λ in brain and 3T3-L1 cells (Fig. 4B and C).

The interaction of ProF with Akt was further demonstrated by a dragging experiment in which a membrane- targeted myristoylated and palmitoylated (m/p)-Akt1 was co-expressed with ProF in COS-7 cells. ProF was able to drag m/p-Akt1 from the plasma membrane to vesicular intracellular structures. (Fig. 5A top, left and right).

Deletion of the FYVE domain induced the opposite effect, namely dragging of ProFΔFYVE by m/p-Akt1 to the plasma membrane (Fig. 5A, bottom, arrow). This experiment further substantiates our finding that the FYVE domain is essential for targeting ProF to internal vesicles. It also shows that the binding of ProF to Akt is sufficient to overcome anchoring of m/p-Akt to the cell surface membrane.

Is has been published previously that insulin stimulation in adipocytes leads to activation of Akt2 and PKCζ/λ and to translocation of GLUT4 vesicles to the plasma membrane (21). We used these cells as model system to study the role of ProF. For that purpose we stably transduced 3T3-L1 cells with a retroviral vector encoding Myc-ProF (pRTP-MycProF) for expression of Myc-tagged ProF in addition to endogenous ProF. These cells were differentiated into adipocytes and analysed by confocal immunofluorescence microscopy. We tested whether Myc-ProF colocalised with Akt2, PKCζ/λ, or GLUT4 without and with insulin stimulation. Without insulin stimulation Akt2 and PKCζ/λ were detected mainly in the cytoplasm, and showed some colocalisation with ProF on punctuate structures (Fig. 5B, C, D). After insulin stimulation for 15 minutes we detected Myc-ProF, the kinases and GLUT4 also at the plasma membrane (Fig. 5B, C, D). These results indicate that ProF is translocated to the plasma membrane in response to insulin, parallel to PKCζ/λ, Akt, and GLUT4.

In order to verify these observations we used a cell fractionation analysis to test the insulin-dependent distribution of endogenous ProF in response to insulin. Unstimulated and insulin-treated differentiated adipocytes were lysed and the localisation of ProF was analysed using differential centrifugation. ProF was detected in the low density microsomal (LDM) fraction in unstimulated cells (Fig. 5E), whereas in stimulated cells it was found in the plasma membrane (PM) fraction. Furthermore, in response to insulin PKCζ/λ and activated Akt were enriched at the PM, and GLUT4

accumulated in the PM. In the cytoplasmic fraction the recruitment of PKCζ/λ to LDMs and PM upon stimulation was reflected by the reduction of the kinase level. This was not observed for activated Akt and total Akt, most likely because the levels of Akt were too high to allow observation of decreased protein levels in the cytoplasmic fraction. GLUT4 was not detected in the cytoplasm and ProF was observed only in very small amounts after stimulation. In conclusion, these results confirm that ProF, PKCζ/λ, activated Akt and GLUT4 move from the vesicle- containing LDM fraction to the plasma membrane upon insulin stimulation.

To further investigate the biological role of ProF, we investigated glucose uptake in 3T3-L1 adipocytes as a model system using overexpressed ProF. Viral transduction of early passage 3T3-L1 cells yielded a pool of Myc-tagged ProF expressing cells, therefore excluding the possibility of clonal variation. The transduced cells were differentiated into adipocytes and glucose uptake was determined after 20 hours of serum- starvation. Glucose uptake was increased by 53% due to overexpression of ProF in the absence of insulin. Using increasing concentrations of insulin for stimulation, presence of Myc-ProF caused an increase of glucose uptake from 48% to 100% (Fig. 6A). Protein levels of endogenous ProF or the unrelated kinase Erk2 were not affected by Myc-ProF as shown by Western Blot (Fig. 6A, bottom). These results demonstrate the contribution of overexpressed ProF to glucose uptake.

To substantiate these results, we performed knock down of Prof. We stably transfected 3T3-L1 cells with lentiviral vectors expressing various siRNAs targeting ProF. siProF1 was targeted against a sequence in the 3'-untranslated region of ProF mRNA, siProF3 against exon 11 of the ProF open reading frame. siProF3 led to almost complete downregulation of endogenous ProF protein levels, whereas siProF1 downregulated the protein only partially (Fig. 6B, top lane). None of the siRNAs affected expression levels of the unrelated protein tubulin (Fig. 6B, bottom lane). Transduction efficiencies were around 95% of the cells. To investigate the role of ProF in adipocytes, we used untransduced, parental 3T3-L1 cells (-), and pools of stably lentivirus- transduced 3T3-L1 cells expressing siProF1 and siProF3, as well as an unrelated siRNA against luciferase (siGL2) as control. At day four after removal of the differentiation cocktail, all cells displayed comparable levels of differentiation, as shown by cell morphology and lipid droplet appearance (Fig. 6B, bottom) and expression of adipocyte-related marker proteins (data not shown). To measure glucose uptake, cells were serum-starved for 20 hours, insulin was added for 30 minutes and then radioactive deoxyglucose was added for 10 minutes. Cells were afterwards lysed and subjected to scintillation counting analysis. The data show that insulin-dependent deoxyglucose uptake was reduced by 55% in siProF3- expressing adipocytes and siProF1 reduced the glucose uptake by 30% in comparison to non-transduced cells, whereas the control siRNA decreased glucose uptake by only 7.5%. The reduction of glucose uptake was dependent on ProF-levels, since more efficient knockdown led to more decreased glucose uptake. These results are in good agreement with the increased insulin-stimulated glucose uptake observed in adipocytes with overexpressed Myc- ProF (Fig. 6A).

Discussion

We have identified a WD-repeat protein containing a FYVE domain, which interacts specifically with the kinases Akt and PKCζ/λ. The putative propeller structure, formed by the WD-repeats, preferentially binds to the activated kinases. This is in accordance with the function of several WD-repeat proteins that also favour binding of activated kinases such as RACK1 and RACK2. RACK1 selectively binds activated PKCβII to enable the interaction of the kinase with its substrates (4). Furthermore, RACK1 is found to associate with integrins, where it regulates focal adhesion and migration by recruiting Src (44). RACK2 recruits activated PKCε to Golgi membranes to control secretion and vesicular

trafficking (46). These examples show that WD-repeat proteins can bring together various kinases as well as kinases and their substrates to integrate signaling pathways. Due to localisation motifs present on WD-repeat proteins, these events can be targeted to specific compartments in the cell.

ProF contains a unique FYVE domain, which was described as a high-affinity PI3P-binding site in the homologous protein FENS-1 (41). We showed that this domain is responsible for the localisation of ProF to internal vesicles. Tissue expression data showed that ProF is expressed in various tissues with high secretory activity such as brain and pancreas (unpublished data). However, in these tissues the involvement of Akt and PKCζ regarding secretory processes is unclear. In ß-pancreatic cells glucose-dependent secretion of insular granules is linked to other PKC isoforms, which do not interact with ProF (unpublished observation), while PKCζ migrates to the nucleus in response to glucose stimulation (47). In the brain, PKC-mediated vesicular trafficking, leading to neurotransmitter release, plays an important role, but the contribution of Akt and PKCζ is unclear.

In contrast, adipocytes have a vesicular trafficking system for insulin-dependent glucose uptake that has been shown to depend on the activation of Akt and PKCζ/λ (for review see (21)). Due to the vesicular localisation of ProF, the binding of kinases Akt and PKCζ, and the high protein expression levels of ProF in these cells (Fig. 2B) we chose adipocytes as a model system to further characterise the role of ProF. Confocal immunofluorescence analysis and cell fractionation revealed that upon insulin stimulation ProF, Akt2, PKCζ/λ, and GLUT4 translocate to the plasma membrane in differentiated 3T3-L1 cells. Overexpression of ProF led to increased and knockdown to decreased glucose uptake in adipocytes. These data suggest that ProF plays a role in general GLUT4 vesicle trafficking. Interestingly, ProF does not only positively influence insulin-stimulated, but also basal glucose uptake. This might be directly connected with the capability of ProF to selectively bind activated Akt and PKCζ/λ. Basal levels of activated kinases exist in unstimulated cells. Furthermore, ProF contains a high-affinity PI3P-binding FYVE site (41) and low levels of PI3P are found at the plasma membrane of unstimulated adipocytes (48), which may be sufficient to recruit some ProF to the plasma membrane in the absence of a stimulus. Under basal conditions a minority of GLUT4 (around 5%) is also found at the plasma membrane of adipose and muscle cells. Thus, basal levels of activated kinases and PI3P in unstimulated cells might be sufficient for ProF to maintain some glucose uptake in 3T3-L1 cells.

Confocal immunofluorescence analysis and cell fractionation revealed that upon insulin stimulation ProF, Akt2, PKCζ/λ, and GLUT4 accumulate at the plasma membrane. The translocation of ProF may be mediated by the FYVE domain, which binds PI3P. PI3P has recently been described to be generated at the plasma membrane of adipocytes upon insulin stimulation (48) and to play a role in translocation of GLUT4-containing vesicles to the plasma membrane. The GLUT4 translocation to the plasma membrane upon incubation of cells with PI3P did not result in fusion of GLUT4 vesicles with the plasma membrane, suggesting that important activation steps leading to vesicle fusion and subsequently to glucose uptake seemed to be missing (49). We have preliminary data supporting this (unpublished observation). It is possible that kinases are required for the missing step, the fusion of the GLUT4 vesicle to the plasma membrane. These kinases may be Akt2, and PKCζ/λ. Thus, ProF may bind via its FYVE- domain in the proximity to the plasma membrane and the kinases may contribute to the next steps.

An important task in respect to the function of ProF as interaction partner for kinases involved in vesicular trafficking would be to determine the substrates of these kinases. A putative PKCζ substrate involved in insulin dependent glucose uptake is the SNARE protein, the vesicle-associated membrane protein 2 (VAMP2), responsible for docking of GLUT4 vesicles at the plasma membrane (50) and the protein 80K-H, which has been

suggested to regulate the fusion of SNARE proteins (51). Akt substrates are AS160, a Rab-GTPase activating protein, which participates in the translocation of GLUT4-containing vesicles to the plasma membrane (52-54) and PIKfyve, which has been implicated in insulin-dependent GLUT4 translocation (55). Presently, we are investigating the role of ProF for the phosphorylation of possible substrates of Akt and PKCζ.

Tissue expression data indicate that ProF might participate in other vesicular trafficking systems. For example the protein PIKfyve, which regulates GLUT4 trafficking in adipocytes (55), is also known to play a role in sorting cargo molecules between the late endosome and the lysosome (56). ProF may be involved in the brain and the pancreas, and it will be interesting to analyse the role of ProF and the kinases involved in these vesicular systems.

In summary, we demonstrated that the WD-FYVE protein ProF serves as a binding platform for the activated kinases Akt and PKCζ. The FYVE domain targets the interacting proteins to vesicular membranes. Our data show that in adipocytes ProF, Akt, PKCζ/λ, and GLUT4 are detected on internal vesicles and after insulin stimulation all proteins accumulate at the plasma membrane. Overexpression of ProF led to increased glucose uptake, while knockdown of ProF by siRNA led to reduced glucose uptake. Furthermore, ProF may be more generally involved in a variety of vesicular trafficking processes in adipocytes or other tissues and may bind to other signaling proteins.

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Footnotes

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The abbreviations used are: AC, accession number; DL, direct lysate; EEA1, early endosomal antigen 1; FENS-1, FYVE domain protein localised to endosomes 1; FYVE, domain identified in Fab1p, YOTB, VAC1p, and EEA1; GLUT4, glucose transporter type 4; IB, immunoblot; IGF-1, insulin-like growth factor: IP, immunoprecipitation; (m/p)-Akt1, myristoylated and palmitoylated Akt1; PDK1, phosphoinositide-dependent kinase 1; PI3P, phosphatidylinositol- 3- phosphate; PI3K, phosphoinositide-3-kinase; PIP_3, phosphatidylinositol- 3,4,5- triphosphate; PKB, protein kinase B; PKC, protein kinase C; ProF, propeller FYVE protein; RACK, receptor of activated C kinases; siRNA, small interfering RNA; TfR, transferrin receptor.

Figure legends

Fig. 1. Characterisation of ProF. (A) Deduced amino acid sequences of human (h) ProF (accession number (AC): AAL04162) and the murine (m) homologue (AC: NP_780755), differing in eight indicated amino acids, are shown. The protein contains seven WD-repeats (WD 1–7), indicated as coloured boxes, and one FYVE domain, indicated as black dashed box. (B) The domain structure of ProF is shown, with the FYVE domain and seven WD domains (WD1-7) indicated. Molecules structurally related to human ProF are found in human (FENS-1, AC: XP_018197), mouse (mProF;

AC: NP_780755; mFENS-1, AC: AAH25226.1), *Drosophila* (AC: AAF52946.1), and *C. elegans* (AC: NP_495983.1). Their amino acid sequence identity with human ProF is given in percent. Scale bar indicates 100 amino acids. (C) The amino acid sequences of the WD-repeats 1 through 7 of ProF are listed. A typical WD-repeat unit contains four ß-strands ßD ßA ßB and ßC (arrows) and consists of about 40 amino acids. Mismatches to a postulated WD-repeat consensus sequence are grey-shaded and their numbers are listed in brackets. The FYVE domain is located between WD6 and WD7 and is indicated by Δ. (D) A model of the three-dimensional structure of ProF without the FYVE domain was generated by the 3D-PSSM program with the yeast protein Tup1 as template. The resulting ß-propeller consists of seven blades formed by the WD-repeats (1-7), whereby the D-strand of each WD-repeat is part of the previous blade (colours are matching Fig. 1A). The FYVE domain is located between the ß-strands 6C and 7D (arrow). (E) The amino acid sequences of the FYVE domains of ProF, FENS-1, and EEA1 are aligned and compared with the predicted secondary structure of EEA1 containing four ß-strands and two α-helices α2 and α3 (top). Highly conserved sequences are R-R-H-H-C-R, W-X-X-D, C-G, R-V-C, and the eight cysteines. The FYVE domains of ProF and FENS-1 have a homologous insert of 11 amino acids and a R to Q mutation in the conserved R-R-H-H-C-R region, indicated in yellow.

Fig. 2. Expression and subcellular localisation of ProF. (A) mRNA expression levels in different cells were analysed by RT-PCR from total RNA with primers specific for murine and human (m/h) ProF as indicated. ß-actin expression was used as control. (B) ProF protein expression levels in different cell lines were analysed by Western blot with an peptide antibody raised against the 15 C-terminal amino acids of ProF and recognising human and mouse ProF. siProF (lane 2) shows downregulation of the ProF protein in 3T3-L1 cells using a siRNA targeted against ProF. Positive (pos.) control (lane 4) indicates a lysate of HEK 293T cells overexpressing untagged ProF. In the case of lane 4, only 1/20 of the protein content of lanes 1 – 3 was loaded to avoid excess signal strength by detection. (C) HeLa cells were transiently transfected with Myc-ProF and subjected to confocal immunofluorescence microscopy with antibodies against the Myc epitope, EEA1, and TfR. Colocalisation of Myc-tagged ProF (red) with endogenous EEA1 (green) on early endosomes and with endogenous TfR (green) on recycling endosomes was visualised in yellow in the merged pictures. Scale bar indicates 20 µm. (D) Immunofluorescence microscopy analysis of COS-7 cells transiently transfected with plasmid DNA encoding Myc-ProF, Myc-ProFΔFYVE, and GFP-ProF. Cells were treated for 30 minutes with 250 µM TPEN or 100 nM wortmannin. Scale bars indicate 20 µm. (E) Oligomerisation: Lysates prepared from HEK 293T cells transiently transfected with the indicated constructs were subjected to immunoprecipitation (IP) with an antibody against the Myc epitope tag. Dimerisation capabilities of ProF and ProFΔFYVE were assessed by immunoblotting (IB) with an antibody against the Flag epitope and by SDS-PAGE (upper panel). The two lower panels show controls for protein expression levels of Myc- and Flag- tagged proteins.

Fig. 3. Interaction of ProF with overexpressed Akt and PKCζ. (A) HEK 293T cells were transiently transfected with HA-Akt1, HA-Bcr, HA-PKCζ, and Src in the presence or absence of Myc-ProF. Interaction of ProF with HA-Akt1, HA-Bcr, HA-PKCζ, and Src was analysed by IP with an antibody to Myc-tag followed by IB with antibodies against Src-, HA-, and Myc-epitopes. Direct lysates are shown as expression controls (bottom). (B) For mapping of the interaction sites of PKCζ and Akt on ProF, HEK 293T cells were transiently transfected with the indicated Myc-ProF constructs and HA-PKCζ (left) and HA-Akt1 (right). The interaction of ProF deletion mutants with PKCζ and Akt was analysed by IP with an antibody to the Myc-tag, followed by IB with antibodies against HA- and Myc-epitopes. The two upper panels show the interaction, the two lower ones show direct lysates as expression controls.

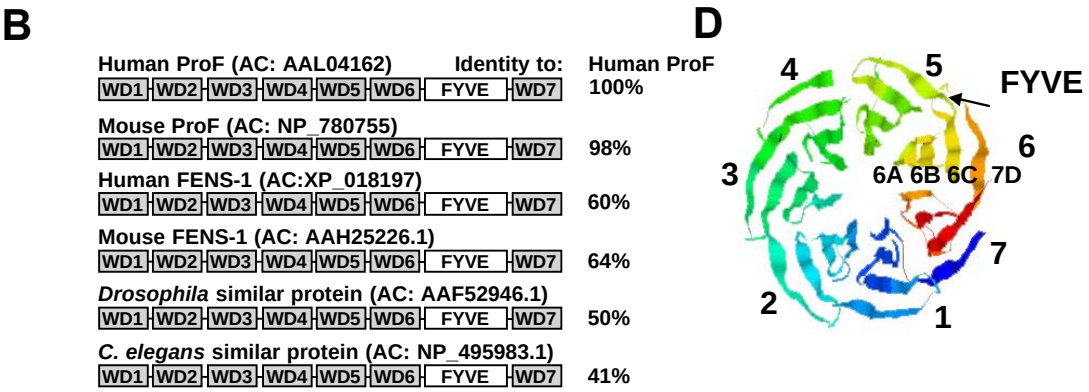
Fig. 4. Half-endogenous and endogenous interactions of ProF with Akt and PKCζ/λ. (A) HEK 293T cells transiently expressing Myc-ProF were stimulated with 100 ng/ml IGF-1 and subjected to IP with an antibody towards the Myc- epitope. Association of Myc-ProF with endogenous Akt1/2 was detected by an anti-Akt antibody and phosphorylation of Akt by an antibody to phospho-Ser 473, indicating increased coimmunoprecipitation of activated ProF after stimulation by IB analysis. Detection of coimmunoprecipitated PKCζ/ι with an antibody against PKCζ/ι shows that the interaction of ProF with PKCζ/ι is increased upon IGF-1 stimulation. The kinase activity of the coprecipitated PKCζ/ι was tested with a PKC substrate peptide. Expression and immunoprecipitation of Myc-ProF was verified by IB of direct lysates and IP with an antibody against the Myc epitope. (B) Mouse brain extract was incubated with anti-ProF antibody in the absence or presence of an excess of ProF peptide to compete for the antibody followed by IB. IB against ProF shows competition of the ProF protein (lane 2). (C) 3T3-L1 pre- adipocyte cells were subjected to IP with an antibody against ProF, crosslinked to sepharose beads with dimethylpimelimidate (lane 3, bottom). Association of endogenous PKCζ/λ with ProF was detected by IB (lane 3, top). Lane 1 shows the position of PKCζ/λ and ProF, using a lysate of HEK 293T cells overexpressing untagged ProF. Lane 2 shows the direct lysate of 3T3-L1 preadipocytes.

Fig. 5. Trafficking and cotranslocation. (A) Dragging. COS-7 cells were transiently transfected with HA-m/p-Akt1 alone and together with Myc-ProF or Myc-ProFΔFYVE. Confocal microscopy analysis with HA- or Myc-specific antibodies revealed areas of colocalisation as visualised in yellow on the merged pictures. Arrow heads indicate colocalisation at the plasma membrane. Scale bar indicates 20 μm. (B, C, D) Colocalisation of ProF with Akt2 (B), PKCζ/λ (C) and GLUT4 (D) Differentiated 3T3-L1 adipocytes (day 15 after removal of hormonal differentiation medium) stably transduced with a retroviral vector encoding Myc-ProF were serum-starved for 2 hours and stimulated with 100 nM insulin as indicated. Cells were subjected to confocal immunofluorescence microscopy using Akt2-, PKCζ/λ-, or GLUT4- (green) and Myc- (red) specific antibodies. Scale bars indicate 20 μm. (E) Differentiated adipocytes (day 15) were serum-starved for 4 hours, stimulated with 100 nM insulin as indicated and fractionated by differential centrifugation. Equal amounts of proteins of cytoplasmic, low density microsomal (LDM), and plasma membrane (PM) fractions were analysed by SDS-PAGE and IB with antibodies against ProF, PKCζ/λ, phospho-Akt1/2, Akt1/2, and GLUT4.

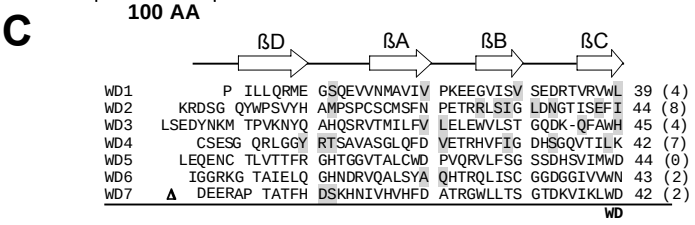
Fig. 6. Effects of ProF overexpression and knock- down on GLUT4 translocation and glucose uptake. (A) Differentiated adipocytes (day 15 after removal of hormonal differentiation medium) overexpressing Myc-ProF (filled columns) or empty vector control (open columns) were starved for 20 hours and stimulated for 1 hour with insulin in the presence of ^{14}C-glucose. Data are mean values ± s.d. of 3 experiments. $*P < 0.05$, $**P < 0.01$ of cells overexpressing Myc-ProF relative to empty vector control (top). Expression of endogenous ProF- protein and overexpressed Myc-ProF protein was analysed by IB (bottom). (B) 3T3-L1 adipocytes were untransduced (-), transduced with the retroviral vector FUGW with insert for expression of siRNA against firefly luciferase as control (siGL2), or with two different inserts for expression of different siRNAs targeted against ProF mRNA (siProF1, siProF3). Adipocytes (day four after removal of hormonal differentiation medium) were starved for 20 hours and stimulated for 30 minutes without (open columns) or with 100 nM insulin (filled columns) before incubation with deoxyglucose for 10 min. Data are mean values ± s.d. of 3 experiments. $*P < 0.05$, $**P < 0.01$ relative to siGL2 cells (top panel). Different levels of ProF expression were analysed by IB (top lane), total protein levels were standardised to tubulin (middle lane). Morphology of the adipocytes was analysed by light microscopy (bottom lane).

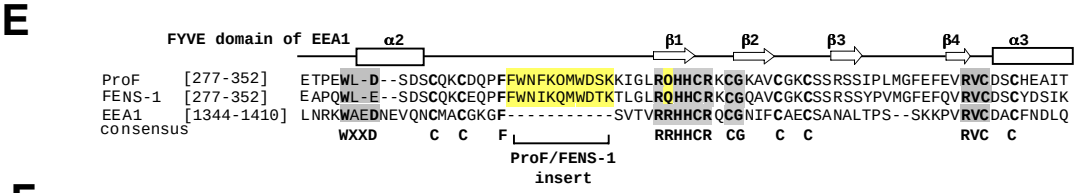
Fig. 1


A
WD1
WD2
WD3
WD4
WD5
WD6
FYVE
WD7


B
Human ProF (AC: AAL04162)
Identity to:
Human ProF
100%
Mouse ProF (AC: NP_780755)
98%
Human FENS-1 (AC:XP_018197)
60%
Mouse FENS-1 (AC: AAH25226.1)
64%
Drosophila similar protein (AC: AAF52946.1)
50%
C. elegans similar protein (AC: NP_495983.1)
41%
100 AA

D
FYVE
1
2
3
4
5
6
6A 6B 6C
7D
7


C
ßD
ßA
ßB
ßC
WD1 P ILLQRME GSQEVVNMAVIV PKEEGVISV SEDRTVRVWL 39 (4)
WD2 KRDSG QYWPSVYH AMPSPCSCMSFN PETRRLSIG LDNGTISEFI 44 (8)
WD3 LSEDYNKM TPVKNYQ AHQSRVTMILFV LELEWVLST GQDK-QFAWH 45 (4)
WD4 CSESG QRLGGY RTSAVASGLQFD VETRHVFIG DHSGQVTILK 42 (7)
WD5 LEQENC TLVTTFR GHTGGVTALCWD PVQRVLFSG SSDHSVIMWD 44 (0)
WD6 IGGRKG TAIELQ GHNDRVQALSYA QHTRQLISC GGDGGIVVWN 43 (2)
WD7 Δ DEERAP TATFH DSKHNIVHVHFD ATRGWLLTS GTDKVIKLWD 42 (2)
WD


E
FYVE domain of EEA1
α2
β1
β2
β3
β4
α3
ProF [277-352] ETPEWL-D--SDSCQKCDQPFFWNFKQMWDSKKIGLRQHHCRKCGKAVCGKCSSRSSIPLMGFEFEVRVCDSCHEAIT
FENS-1 [277-352] EAPQWL-E--SDSCQKCEQPFFWNIKQMWDTKTLGLRQHHCRKCGQAVCGKCSSRSSYPVMGFEFQVRVCDSCYDSIK
EEA1 [1344-1410] LNRKWAEDNEVQNCMACGKGF----------SVTVRRHHCRQCGNIFCAECSANALTPS--SKKPVRVCDACFNDLQ
consensus
WXXD C C F RRHHCR CG C C RVC C
ProF/FENS-1
insert

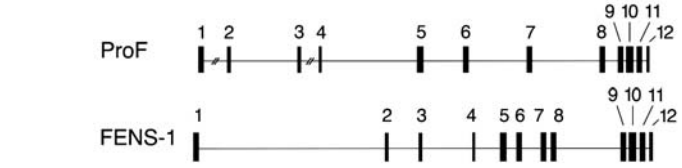

F
ProF
FENS-1
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2

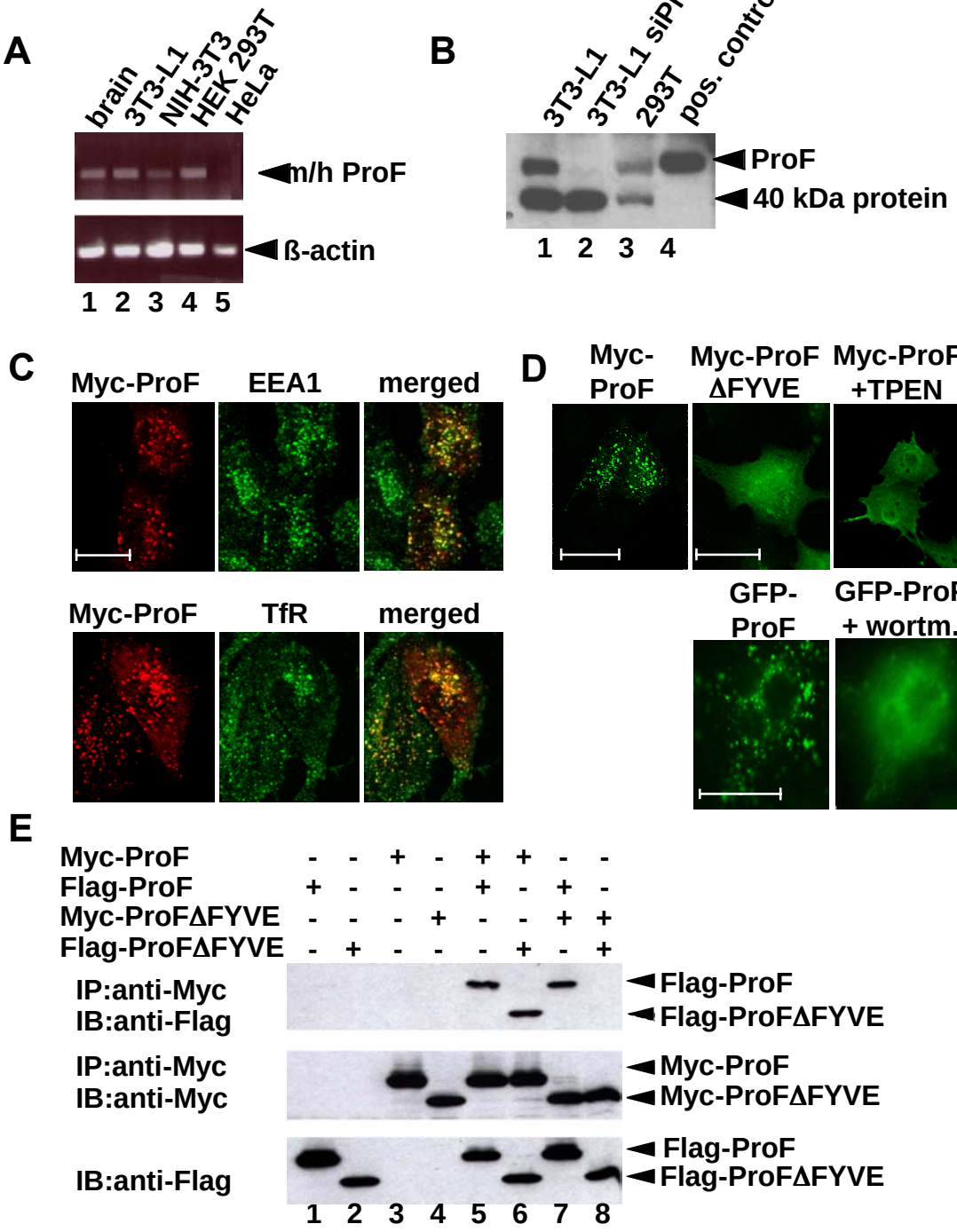

A
brain
3T3-L1
NIH-3T3
HEK 293T
HeLa
m/h ProF
ß-actin
1 2 3 4 5
B
3T3-L1
3T3-L1 siProF
293T
pos. control
ProF
40 kDa protein
1 2 3 4
C
Myc-ProF
EEA1
merged
Myc-ProF
TfR
merged
D
Myc-ProF
Myc-ProF ΔFYVE
Myc-ProF +TPEN
GFP-ProF
GFP-ProF + wortm.
E
Myc-ProF - - + - + + - -
Flag-ProF + - - - + - + -
Myc-ProFΔFYVE - - - + - - + +
Flag-ProFΔFYVE - + - - - + - +
IP:anti-Myc
IB:anti-Flag
Flag-ProF
Flag-ProFΔFYVE
IP:anti-Myc
IB:anti-Myc
Myc-ProF
Myc-ProFΔFYVE
IB:anti-Flag
Flag-ProF
Flag-ProFΔFYVE
1 2 3 4 5 6 7 8

Fig. 3

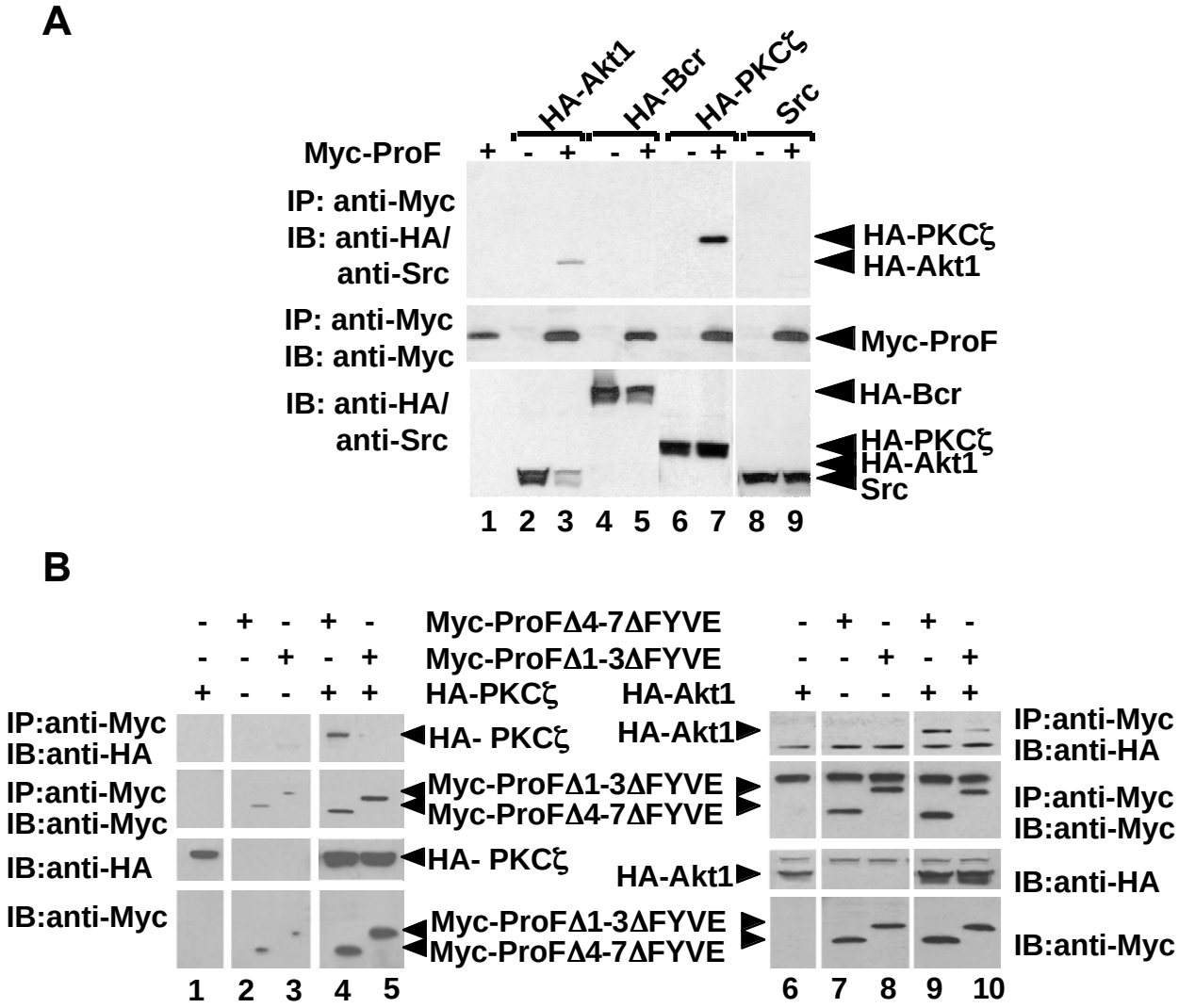

A
HA-Akt1
HA-Bcr
HA-PKCζ
Src
Myc-ProF + - + - + - + - +
IP: anti-Myc
IB: anti-HA/
anti-Src
HA-PKCζ
HA-Akt1
IP: anti-Myc
IB: anti-Myc
Myc-ProF
IB: anti-HA/
anti-Src
HA-Bcr
HA-PKCζ
HA-Akt1
Src
1 2 3 4 5 6 7 8 9
B
Myc-ProFΔ4-7ΔFYVE
Myc-ProFΔ1-3ΔFYVE
HA-PKCζ
HA-Akt1
IP:anti-Myc
IB:anti-HA
HA- PKCζ
HA-Akt1
IP:anti-Myc
IB:anti-Myc
Myc-ProFΔ1-3ΔFYVE
Myc-ProFΔ4-7ΔFYVE
IB:anti-HA
HA- PKCζ
HA-Akt1
IB:anti-Myc
Myc-ProFΔ1-3ΔFYVE
Myc-ProFΔ4-7ΔFYVE
1 2 3 4 5 6 7 8 9 10

Fig. 4

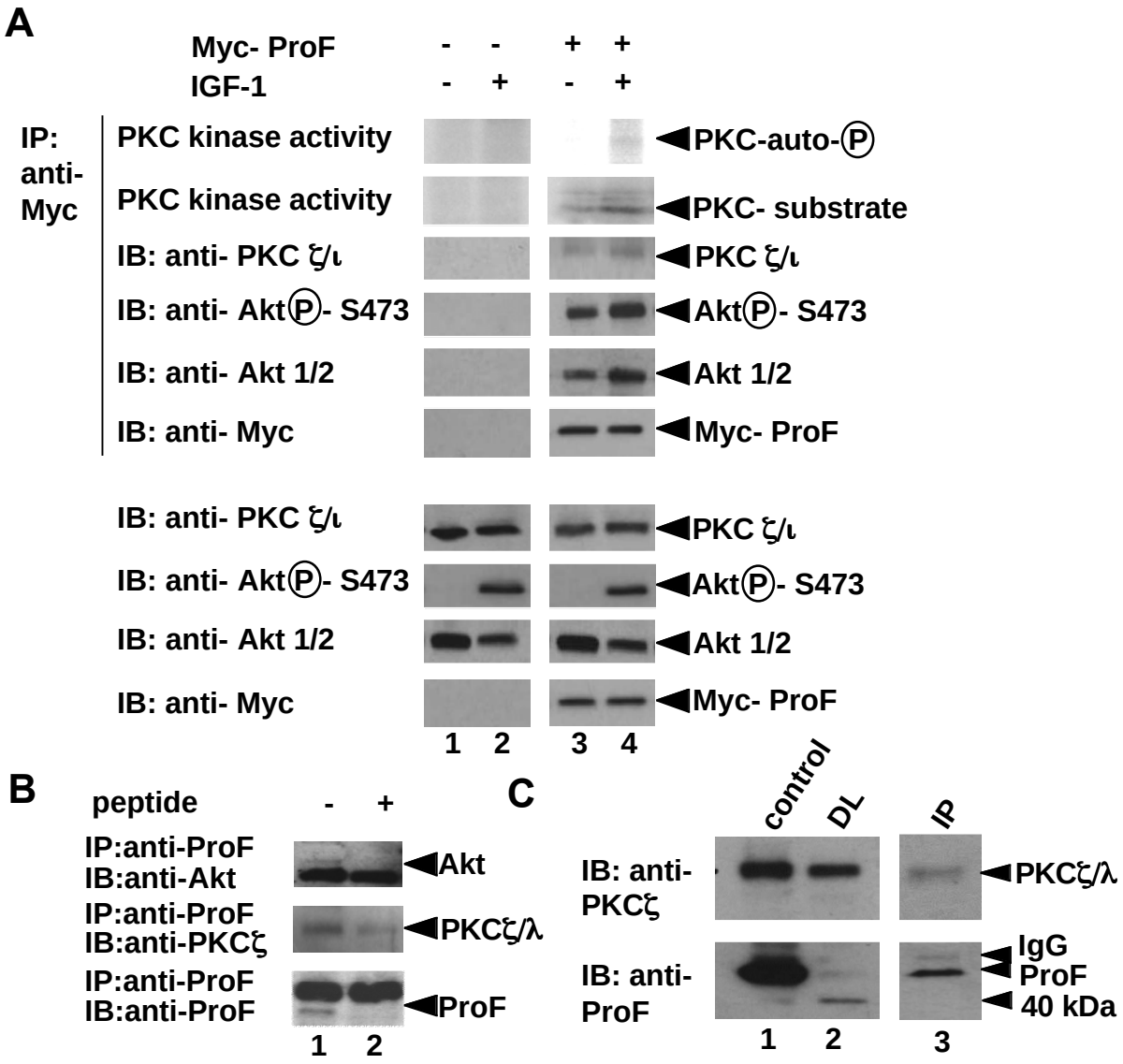

A
Myc- ProF - - + +
IGF-1 - + - +
IP: anti-Myc
PKC kinase activity
PKC-auto-Ⓟ
PKC kinase activity
PKC- substrate
IB: anti- PKC ζ/ι
PKC ζ/ι
IB: anti- AktⓅ- S473
AktⓅ- S473
IB: anti- Akt 1/2
Akt 1/2
IB: anti- Myc
Myc- ProF
IB: anti- PKC ζ/ι
PKC ζ/ι
IB: anti- AktⓅ- S473
AktⓅ- S473
IB: anti- Akt 1/2
Akt 1/2
IB: anti- Myc
Myc- ProF
1 2 3 4
B
peptide - +
IP:anti-ProF IB:anti-Akt
Akt
IP:anti-ProF IB:anti-PKCζ
PKCζ/λ
IP:anti-ProF IB:anti-ProF
ProF
1 2
C
control
DL
IP
IB: anti-PKCζ
PKCζ/λ
IB: anti-ProF
IgG
ProF
40 kDa
1 2 3

Fig. 5

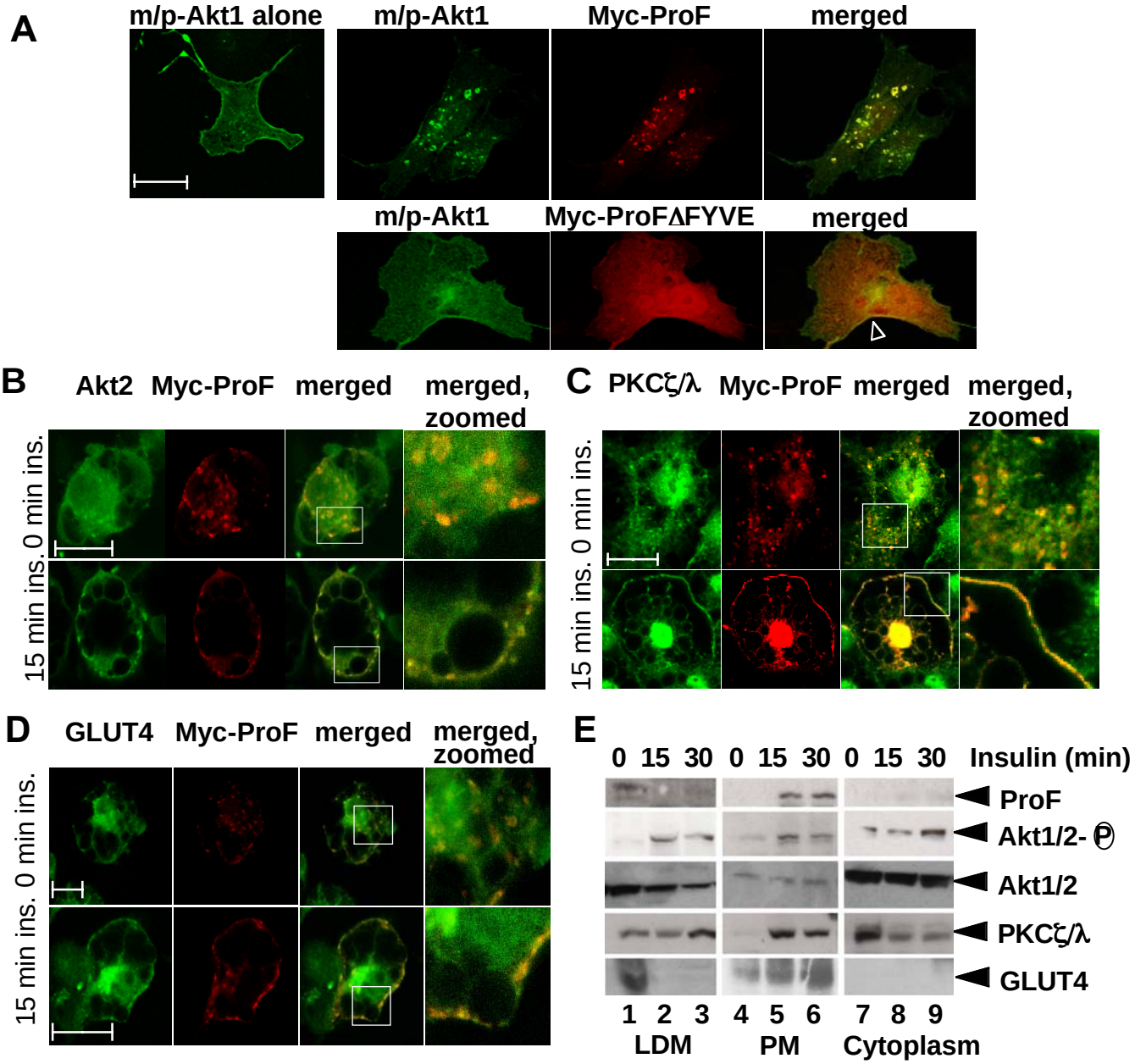
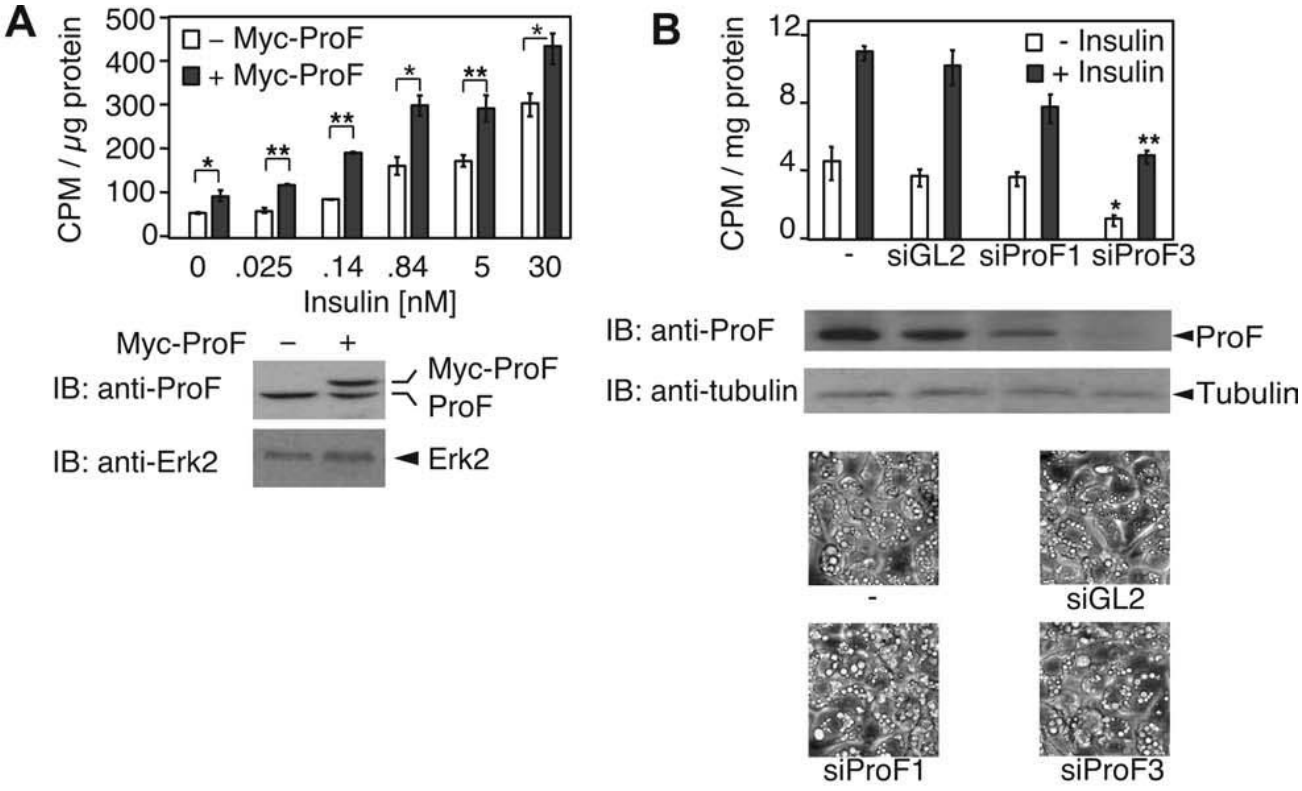

A
m/p-Akt1 alone
m/p-Akt1
Myc-ProF
merged
m/p-Akt1
Myc-ProFΔFYVE
merged
B
Akt2
Myc-ProF
merged
merged, zoomed
15 min ins.
0 min ins.
C
PKCζ/λ
Myc-ProF
merged
merged, zoomed
15 min ins.
0 min ins.
D
GLUT4
Myc-ProF
merged
merged, zoomed
15 min ins.
0 min ins.
E
0 15 30 0 15 30 0 15 30 Insulin (min)
ProF
Akt1/2- P
Akt1/2
PKCζ/λ
GLUT4
1 2 3 4 5 6 7 8 9
LDM
PM
Cytoplasm

Fig. 6


A
CPM / μg protein
500
400
300
200
100
0
– Myc-ProF
+ Myc-ProF
0 .025 .14 .84 5 30
Insulin [nM]
Myc-ProF – +
IB: anti-ProF
Myc-ProF
ProF
IB: anti-Erk2
Erk2
B
CPM / mg protein
12
8
4
0
- Insulin
+ Insulin
- siGL2 siProF1 siProF3
IB: anti-ProF
ProF
IB: anti-tubulin
Tubulin
-
siGL2
siProF1
siProF3

4.2 A WD-FYVE PROTEIN BINDS VAMP2 AND PKCζ AND INCREASES PKCζ-DEPENDENT PHOSPHORYLATION OF VAMP2

(Manuscript submitted)

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Running Title: WD-FYVE protein increases PKCζ-dependent VAMP2 phosphorylation

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Summary

We have recently identified and characterized a protein, consisting of seven WD-repeats, presumably forming a β-propeller, and a FYVE domain (ProF), which targets the protein to vesicular membranes. ProF bound the activated kinases Akt and protein kinase Cζ/λ (PKCζ/λ) upon stimulation with insulin-like growth factor-1 (IGF-1) (1). Here we describe the vesicle-associated membrane protein 2 (VAMP2) as interaction partner of ProF. The interaction was demonstrated with the ectopically expressed and the endogenous proteins in mammalian cells. ProF and VAMP2 colocalized on vesicular structures with PKCζ and the three proteins formed a ternary complex. IGF-1 stimulation of HEK293T cells activated PKCζ, which in turn led to phosphorylation of VAMP2. ProF recruited activated PKCζ to VAMP2, which increased phosphorylation of VAMP2 *in vitro*. By binding PKCζ and VAMP2, ProF can integrate the kinase with its substrate. VAMP2 is known to regulate docking and fusion of vesicles and to play a role in targeting vesicles to the plasma membrane. Thus, ProF may act as adaptor protein for PKCζ and VAMP2 and by this may regulate vesicle cycling. The ternary complex of ProF, VAMP2, and PKCζ may also be involved in other secretory pathways.

Introduction

We have recently identified the propeller-FYVE protein (ProF) as a binding partner for Akt and protein kinase Cζ/λ (PKCζ/λ) (1). ProF contains seven WD-repeats, which form a propeller-like structure providing a protein binding-platform (2). Furthermore, ProF harbors a FYVE domain that specifically interacts with phosphatidylinositol-3-phosphate (PI3P) (3) and targets ProF to internal vesicles. Deletion of the FYVE domain or inhibition of PI3P-formation by a phosphoinositide-3-kinase-inhibitor resulted in loss of vesicular

localization. ProF preferentially bound to the activated kinases Akt and PKCζ/λ upon stimulation with insulin-like growth factor-1 (IGF-1) (1).

In adipocytes ProF translocated with the kinases and glucose transporter 4 (GLUT4) to the plasma membrane in response to insulin stimulation. Akt and PKCζ/λ are known to play a key role in insulin-dependent translocation of GLUT4 from internal storage vesicles to the plasma membrane in adipose tissues (4). We recently demonstrated that overexpression of ProF led to increased glucose uptake upon insulin stimulation, while knock-down of ProF by small interfering RNA (siRNA) led to reduced glucose uptake. This suggests a role of ProF in glucose metabolism and possibly in other secretory pathways, because of its broad tissue expression (1).

In order to better understand the role of ProF in regulated vesicle trafficking, we searched for substrates of Akt and PKCζ on vesicles. While this work was in progress, the Akt substrate of 160 kDa (AS160) has been found to be located on GLUT4-containing vesicles in adipocytes (5) where it affects vesicle trafficking upon Akt phosphorylation (6-8). Several PKCζ substrates have been described previously. The vesicle-associated membrane protein 2 (VAMP2) may be one of them (9). VAMP2 belongs to the vesicular soluble *N*-ethylmaleimide-sensitive fusion protein (NSF)-attachment protein receptors (v-SNARE). This protein family comprises eight members involved in secretory pathways (10). VAMP2 is widely expressed in a large variety of tissues such as brain, kidney, adrenal gland, liver and pancreas (11). VAMP2 is crucial for stimulus-dependent secretion in various cell-types including insulin-stimulated GLUT4 translocation in adipocytes and muscle cells (12-14), general fusion of early and sorting endosomes (15,16), and synaptic vesicle fusion with the plasma membrane in neurons (17-20). The fusion of VAMP2-containing vesicles with the plasma membrane is mediated by complex formation of the v-SNARE with the target-(t) SNAREs, which are the synaptosome-associated protein (SNAP) and syntaxin (21). VAMP2 has previously been shown to be phosphorylated in myotubes overexpressing PKCζ, which correlated with increased GLUT4 translocation and glucose uptake (9). GLUT4 consists of twelve transmembrane spanning α-helix segments forming a central pore for diffusion of glucose molecules into the cell (22).

ProF has been shown in our previous analysis to be a binding partner of PKCζ(1). In the present study we show that ProF also interacts with VAMP2 *in vitro* and *in vivo*. We furthermore demonstrate that all three proteins form a ternary complex. In this complex ProF brings PKCζ in proximity to its substrate VAMP2 and leads to increased phosphorylation of VAMP2 by activated PKCζ. Thus, ProF is an adaptor protein, which integrates kinases and their substrates such as VAMP2, which, upon phosphorylation, may contribute to the regulation of exocytotic processes.

Experimental procedures

Antibodies and reagents - Antibodies against Myc-epitope (A14, rabbit polyclonal and 9E10, mouse monoclonal), against hemagglutinin (HA) (Y-11, rabbit polyclonal), and PKCζ (C-20, rabbit and goat polyclonal), were obtained from Santa Cruz Biotechnology. The mouse monoclonal and the rabbit polyclonal antibody to VAMP2 were from Synaptic Systems and Calbiochem, respectively. The antibody to Flag-epitope (M2, mouse monoclonal) was from SIGMA. A polyclonal peptide antibody directed against the 15 C-terminal amino acids of ProF was raised in rabbits and affinity purified on the peptide used for immunization (1). This peptide was also used for peptide competition in endogenous interaction analysis. All secondary antibodies for immunoblotting and indirect immunofluorescence staining were from Pharmacia Amersham and Jackson Immuno Research, respectively.

Yeast two-hybrid analysis - A human B cell-specific cDNA library was obtained from S.J. Elledge (Baylor College of Medicine, Houston, Texas) (23). The yeast two-hybrid

analysis was performed essentially as described using ProF as a bait (24). The plasmid encoding Flag-VAMP2 has previously been described (25) and was kindly provided by Dr. Mitsunori Fukuda (RIKEN, Japan).

Recombinant DNA manipulation and plasmid constructs - Serine to alanine point mutations were inserted into the coding sequence of Flag-VAMP2 using the Quick Change Mutagenesis Kit (Stratagene). Plasmid pEF-Flag-VAMP2 was used as template. The primers for mutagenesis were obtained from Microsynth (Balgach, Switzerland), mutation 1 (S28A): forward, cca aac ctt act **gct** aac agg aga ctg, reverse, cag tct cct gtt agc agt aag gtt tgg; mutation 2 (S61A): forward, gac cag aag ttg **gcg** gag ctg gat gac, reverse: gtc atc cag ctc cgc caa ctt ctg gtc, mutation 3 (S75A): forward, gca ggg gcc **gcc** cag ttt gaa, reverse: ttc aaa ctg ggc ggc ccc tgc, mutation 4 (S80A): forward, cag ttt gaa aca **gct** gca gcc aag ctc, reverse, gag ctt ggc tgc agc tgt ttc aaa ctg. The inserted mutation is highlighted in bold in the forward primer. Mutants containing four single and one mutant harboring all four mutations were constructed. All mutations were verified by DNA sequencing.

N-terminally Myc-tagged human ProF encoding constructs were used (1): Myc-ProF, Myc-ProFΔFYVE lacking the FYVE domain for PI3P binding, Myc-ProFΔ1-3ΔFYVE lacking blades 1 to 3 and the FYVE domain, Myc-ProFΔ4-7ΔFYVE lacking blades 4 to 7 and the FYVE domain and Myc-ProFΔ4-7 lacking blades 4 to 7 were used in this study.

A HA-tagged PKCζ construct was obtained from F. J. Johannes.

Retroviral transduction and generation of stably transduced 3T3-L1 fibroblasts Retroviruses containing the construct pRTP-Myc-ProF or the empty pRTP vector as control were produced using the BOSC-23 packaging cell-line as described (26,27). Early passage 3T3-L1 fibroblasts were incubated in virus-containing medium for 48 h. The cells were used for immunofluorescence studies.

Immunoprecipitation - For transient expression the human embryonic kidney cell line (HEK) 293T was transfected with expression vectors encoding the indicated proteins using calcium phosphate (28). Cells were lysed using a lysis buffer, containing 100 mM NaCl, 1 mM ethylene-diaminetetraaceticacid (EDTA), 20 mM Tris-HCl, and 0.5 % NP-40 (NETN) (28) including complete EDTA-free protease inhibitor tablets (Roche Medicals) and cleared by centrifugation at 16.000g and 4°C.

Interactions of endogenous proteins were analyzed in mouse brain extracts. Proteins were extracted in an extraction buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 0.2% NP-40 and supplemented with protease inhibitor tablets, by incubating the lysates at 4°C for 30 minutes at vigorous shaking. Glycerol was added to a final concentration of 10% after clearing of lysates. Protein concentrations were determined by Bradford assay in a microtiterplate reader at 595 nm using Bovine serum albumin (BSA) as standard.

Lysates were precleared for 1 hr using 30 µl Protein-A-Sepharose (Amersham Pharmacia Biotech). Cleared lysates were immunoprecipitated with 1 µg of the appropriate antibody for at least 3 h at 4°C and then 1 h in the presence of 10 µl of protein G-sepharose (Amersham Pharmacia Biotech). Immunoprecipitation of ProF was conducted overnight at 4°C. For competition studies 1 µg anti-ProF antibody was preincubated with 10 µg peptide on ice for 30 minutes. Elution of overexpressed Flag-VAMP2 was performed by vigorous shaking for 1 hr at 24°C, followed by two rounds of shaking for 1hr at 4°C in 120 µl of NETN- buffer containing 900 µg/µl of Flag-peptide (Sigma).

Lysates and the resulting immunoprecipitates were resolved on commercial 10-20% SDS-polyacrylamide gradient gels (Invitrogen). The primary and secondary antibodies were used at a dilution of 1:10.000. Immunoblotting of endogenous ProF was performed overnight at 4°C with a 1:1.000 dilution of the antibody. All incubation and wash steps were performed in 1% non-fat dry milk in Tris- buffered saline (TBS) containing 0.1% Tween-20. Detection was performed using a chemoluminescence detection kit (Amersham Pharmacia Biotech).

Confocal microscopy - The simian kidney derived cell line COS-7 was grown on glass cover slips and was transiently transfected with Fugene-6 (Roche Medicals) according to the manufacturers` instructions. Twenty hrs after transfection, cells were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde and permeabilized in PBS containing 0.25% Triton X-100. Cover slips were successively incubated with appropriate primary and secondary antibodies before they were mounted in Mowiol (Hoechst Pharmaceuticals). The cells were examined by sequential excitation at 488 nm (fluorescein isothiocyanate (FITC)), 568 nm (tetramethylrhodamine isothiocyanate (TRITC)) and 633 nm (cyanine-5 (CY5)) using a confocal microscope (SP2, Leica) and a 40x1.25 oil objective (Leica). The images were processed by using Photoshop (Adobe Systems).

In vitro kinase assay - Flag-VAMP2 was transiently expressed in the presence or absence of Myc-ProF in HEK 293T cells. Cells were lysed as described above and lysates were immunoprecipitated using an anti-Flag antibody. Immunocomplexes were washed in a kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM $CaCl_2$, 1 mM DTT). For the kinase reaction the buffer was supplemented with 3 μM protein kinase A inhibitor, 20 μM ATP, and 10 μCi γ-^{32}P-ATP with a specific activity of 3.000 mCi/mole (Amersham Pharmacia). To start the reaction 0.2 μg recombinant PKCζ (Upstate) was added to the samples. The phosphorylation reaction was conducted at 30°C for 30 min and was stopped by boiling the samples in sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were resolved on 10-20% SDS-polyacrylamide gradient gels. Expression of Flag-VAMP2 and Myc-ProF was verified by immunoblotting and phosphorylation was visualized using a Storm 840 Phosphoimager (Molecular Dynamics).

Results

We have recently identified a protein, consisting of seven WD-repeats, presumably folding into a β-propeller-type structure, and a FYVE domain (Fig. 1*A*), designated as ProF. ProF interacted via its WD-repeats with the serine/ threonine kinases Akt and PKCζ/λ and was located on internal vesicles via its FYVE domain (1). These two kinases preferentially bound to ProF in their activated, phosphorylated forms upon IGF-1 stimulation. We furthermore showed that ProF is required for glucose uptake in differentiated adipocytes. Therefore, the question arose whether ProF at the same time interacted also with putative kinase substrate.

In order to identify such candidates, we performed a yeast two-hybrid screen using ProF as a bait and identified VAMP2 as a putative interaction partner. VAMP2 is a v-SNARE protein associated with vesicular membranes via its C-terminal transmembrane domain. Its central SNARE domain of approximately 60 amino acids allows the interaction of VAMP2 with its cognate t-SNARE proteins (Fig. 1*A*) (10).

To verify these results, we first analyzed the interaction of VAMP2 with ProF by coimmunoprecipitation of overexpressed proteins. For that purpose, Myc-tagged ProF and Flag-tagged VAMP2-expression constructs were cotransfected into HEK 293T cells. Cell lysates were treated with an anti-Flag antibody and the precipitates were analyzed by immunoblotting for the presence of coprecipitating proteins. As can be seen, Myc-tagged ProF indeed coprecipitated with Flag-tagged VAMP2 (Fig. 1*B*), validating the results from yeast two-hybrid screen.

Further indications for the interaction of both proteins were obtained by confocal immunofluorescence analysis showing their subcellular distribution. For that, Flag-tagged VAMP2 and Myc-tagged ProF were co-expressed in COS-7 cells and analyzed by confocal microscopy. As can be seen, a partial colocalization of VAMP2 (green signal) and ProF (red signal) on vesicular structures was detectable (Fig. 1*C*). Colocalization of the two proteins is indicated by the orange color, detectable in the merged picture, showing the superposition of the two signals.

We further characterized the interaction between ProF and VAMP2 with deletion mutants of ProF. We designed Myc-tagged mutant ProF proteins, lacking either the FYVE domain or the FYVE domain in conjunction with blades 1 to 3 or blades 4 to 7 of the β-propeller (indicated in Fig. 1*D*). We coexpressed these proteins together with Flag-tagged VAMP2 in HEK 293T cells and tested the interaction by coimmunoprecipitation assays. As can be seen, all ProF-mutants interacted equally well with VAMP2 (Fig. 1*D*). This result suggested that multiple binding sites on ProF are involved in the binding of VAMP2.

We have previously shown that ProF binds PKCζ (1) and show here the interaction between ProF and VAMP2. Furthermore, overexpression of PKCζ has been reported to result in an increased phosphorylation of VAMP2 (9). This raised the question whether ProF would affect the interaction between VAMP2 and PKCζ. In order to test that, we expressed Flag-VAMP2 and HA-PKCζ in the presence or absence of Myc-ProF in HEK 293T cells (Fig. 2*A*). As can be seen, coexpression of Myc-ProF led to the coprecipitation of large amounts of HA-PKCζ by Flag-VAMP2 (Fig 2*A*, lane 6). In the absence of Myc-ProF only small amounts of HA-PKCζ were coprecipitated, indicating a weak interaction (Fig. 2*A*, lane 5), which may be due to endogenous Prof. Furthermore, in the absence of HA-PKCζ only small amounts of Myc-ProF were coprecipitated, indicating that the presence of all three proteins might stabilize the ternary complex. Thus, ProF could serve as an adaptor protein that mediates the binding of PKCζ and VAMP2.

The ternary complex formation was further confirmed by a sequential precipitation procedure. For that purpose, we overexpressed the epitope-tagged forms of all three proteins in HEK 293T cells. First we immunoprecipitated Flag-VAMP2 and showed coprecipitation of Myc-ProF and HA-PKCζ by Western Blotting analysis of an aliquot of the immunoprecipitate (Fig. 2*B*, lane 2). The precipitated complex was thereafter eluted with a Flag-peptide and Myc-ProF was immunoprecipitated. As can be seen, coimmunoprecipitation of HA-PKCζ was demonstrated by immunoblotting (Fig. 2*B*, lane 3). Coimmunoprecipitation of Flag-VAMP2 was not detectable, possibly because VAMP2 dissociated from the complex after elution. Thus, Myc-ProF binds both proteins, Flag-VAMP2 and HA-PKCζ, simultaneously.

To demonstrate the colocalization of all three proteins, we performed confocal microscopy analysis. For that, Flag-tagged VAMP2, HA-tagged PKCζ and Myc-tagged ProF were transiently expressed in COS-7 cells and analyzed by confocal microscopy. As can be seen, a partial colocalization of the three proteins on intracellular vesicles was detected (Fig. 2*C*). Colocalization of the three proteins is indicated by white color in the merged picture, showing the superposition of the three signals.

So far we analyzed overexpressed proteins and now wanted to confirm our results with endogenous proteins. ProF and VAMP2 have been shown to be expressed in the brain (1,29). Therefore, we tested mouse brain lysates for the interaction between endogenous ProF and VAMP2. For that, brain lysates were treated with an anti-ProF antibody with and without peptide competition (1). The peptide competition demonstrated the specificity of the reaction. The precipitates were analyzed by Western Blotting. As can be seen, coprecipitation of VAMP2 with ProF was detectable. As ProF was not precipitated in the presence of the peptide, the loss of the VAMP2 signal in the immunoprecipiate indicates an interaction between ProF and VAMP2 (Fig. 3*A*). Thus, we confirmed the interaction not only for overexpressed ProF and VAMP2, but also for endogenous proteins in brain tissue.

In order to further characterize the interaction of the endogenous proteins, we immunoprecipitated all three endogenous proteins, ProF, VAMP2, and PKCζ from mouse brain lysates. The lysates were treated with antibodies against the individual proteins and the precipitates were analyzed by Western Blotting with antibodies against ProF, VAMP2, and PKCζ. As can be seen, only

after precipitation with anti-ProF antibodies all three proteins were present in the immunoprecipitates (Fig. 3*B*). PKCζ precipitated small amounts of VAMP2, but did not precipitate detectable amounts of ProF, and VAMP2 precipitated small amounts of ProF, but did not precipitate detectable amounts of PKCζ. This result confirms the role of ProF as binding partner for VAMP2 and PKCζ not only in the overexpression system, but also for the endogenous proteins as demonstrated in brain tissue.

We confirmed these biochemical results by confocal microscopy studies with 3T3-L1 cells. For that, 3T3-L1 pre-adipocytes, stably expressing Myc-ProF, were subjected to immunofluorescence analysis. As can be seen, Myc-ProF, endogenous VAMP2, and endogenous PKCζ colocalized on perinuclear vesicular structures (Fig. 3*C*). These results are in agreement with the previous findings that ProF is located on internal vesicles in various cell lines and e.g. in unstimulated 3T3-L1 adipocytes, where it colocalized with endogenous PKCζ (1).

Next we asked whether activated PKCζ was able to directly phosphorylate VAMP2. We considered this as likely, because it had been shown by Braiman and coworkers, that overexpression of wild-type PKCζ in myotubes accelerated insulin-dependent serine phosphorylation of VAMP2, while overexpression of dominant negative PKCζ completely abolished VAMP2 phosphorylation even in the presence of insulin. Thus, these authors supplied some evidence for phosphorylation of VAMP2 by PKCζ (9). We addressed this question by transient overexpression of Flag-VAMP2 with and without HA-PKCζ in HEK 293T cells. Cells were stimulated with 100 ng/ml IGF-1 in order to activate PKCζ, or left unstimulated. 15 min later cells were lysed and Flag-VAMP2 and associated proteins were immunoprecipitated using an anti-Flag antibody. The precipitates were subjected to an *in vitro* kinase assay. Phosphorylation was initiated by adding γ-^{32}P-ATP. Subsequently the precipitates were separated by SDS-PAGE and analyzed for radioactive signals using the PhosphoImager. As can be seen, IGF-1-stimulation led to substrate phosphorylation of the immunoprecipitated VAMP2 and to phosphorylation of the coimmunoprecipitated PKCζ (Fig, 4*A*). Thus, VAMP2 phosphorylation depends on IGF-1 stimulation and activated PKCζ.

After having confirmed the phosphorylation of VAMP2 by IGF-1-stimulated PKCζ, we mapped the phosphorylation site on VAMP2. For that, we generated mutants of VAMP2, in which different serine residues were mutated to alanine. Out of the six serine residues conserved in mouse and rat VAMP2 (S2, S28, S61, S75, S80, S115) (Fig. 1*A*), we excluded S2 from mutation because of its position at the very N-terminus and S115, because of its C-terminal position and its location inside the vesicle, which seemed to be an unlikely target for phosphorylation. The four remaining serine residues were mutated to alanine. Three of these sites are located within the SNARE motif (S61, S75 and S80). The fourth one is located in the N-terminal sequence (S28) and has previously been reported to represent a PKC phosphorylation site *in vitro* (30). The only mutant that showed a markedly reduced ^{32}P-phosphorylation by about 70% reduction after PKCζ treatment was the Flag-VAMP2mt(1-4) mutant, in which all four possible PKC targets were converted into alanine (data not shown). Residual ^{32}P-phosphorylation could be due to phosphorylation of threonine residues by PKCζ. This indicated that all four serine residues of VAMP2 could be PKCζ-dependent phosphorylation sites

So far we have shown that VAMP2 is a substrate of PKCζ in HEK 293T cells upon IGF-1 stimulation (Fig. 4*A*). In a final experiment we wanted to include the analysis of ProF and its effects. Instead of using IGF-1-stimulated cells we treated coimmunoprecipitates of VAMP2 and ProF with active recombinant PKCζ, because we have shown above that ProF can lead to increased binding of PKCζ to VAMP2 (Fig. 2*A*). Therefore, it appeared likely that this interaction resulted in increased

phosphorylation of VAMP2. In order to test this, Flag-VAMP2 wild-type (wt) and the Flag-VAMP2 mutant with the four S to A mutations, designated as Flag-VAMP2mt(1-4), were transiently expressed with and without Myc-ProF in HEK 293T cells. Flag-VAMP2 was immunoprecipitated with an anti-Flag antibody. The precipitates were subjected to an *in vitro* kinase assay using recombinant active PKCζ and subsequently analyzed for the presence of ^{32}P-phosphorylation. As expected, phosphorylation of Flag-VAMP2 by PKCζ was increased in the presence of Myc-ProF (Fig. 4*B*). Flag-VAMP2mt(1-4) did not show *in vitro* ^{32}P-phosphorylation (Fig. 4*B*, lane 3), proving the specificity of the interaction. In summary, these data revealed that the presence of Myc-ProF increases the *in vitro* phosphorylation of Flag-VAMP2 by activated PKCζ.

Discussion

We have previously identified ProF as an adaptor molecule, which is located on internal vesicles and binds to activated Akt and PKCζ upon IGF-1-stimulation (1). This raised the question on putative kinase substrates, which might also interact with ProF. We addressed this question using different experimental approaches. An initial yeast two-hybrid screen indicated the possibility of VAMP2 being a binding partner of ProF. We confirmed the physical interaction of VAMP2 and ProF by coimmunoprecipitation of overexpressed and endogenous proteins. VAMP2 is known to be anchored via its transmembrane domain to secretory vesicles in e.g. adipocytes where it represents the v-SNARE protein responsible for mediating fusion of vesicles, such as the insulin-induced fusion of GLUT4–containing vesicles with the plasma membrane (13,31). In adipocytes and skeletal muscle cells, VAMP2 has been described to bind to the t-SNARE proteins syntaxin-4 and SNAP-23, found at the plasma membrane (32,33), whereas in neurons VAMP2 interacts with syntaxin-1 and SNAP-25 at the plasma membrane for neurotransmitter release (17-20). These findings demonstrate the general role of VAMP2 in a number of secretory systems.

Many vesicle cycling events rely on the interaction of v-SNARE and t-SNARE proteins, which allows docking of vesicles to their target membranes. SNARE complex formation is thought to bring the opposing membranes close enough for fusion (34). These SNARE-dependent fusion events include a number of secretory processes such as insulin release from pancreatic β-cells (35-37), synaptic vesicle exocytosis (38), granule release in hematopoetic cells (39), and aquaporin- (40), or GLUT4-translocation to the plasma membrane (14,41). In general, exocytotic events are regulated by a variety of mechanisms including phosphorylation of SNARE and accessory proteins (38).

In this study we show that ProF can mediate the interaction between PKCζ and VAMP2. ProF, VAMP2, and PKCζ colocalized on vesicular structures and formed a ternary complex. Therefore, we hypothesized that the presence of ProF may improve phosphorylation of VAMP2. Indeed, PKCζ-dependent phosphorylation of VAMP2 *in vitro* was enhanced in the presence of ProF. Furthermore, IGF-1 stimulation induced activation of PKCζ and phosphorylation of VAMP2 *in vitro*. It is currently unknown how the PKCζ-dependent phosphorylation influences the interaction of VAMP2 with its cognate t-SNAREs or with accessory proteins. Whether the PKCζ-dependent phosphorylation of VAMP2 decreases or increases the interaction between the v-SNARE and t-SNARE proteins and how it regulates vesicle cycling should be investigated in future studies.

We specified four serine residues within the VAMP2 molecule, which represent potential phosphorylation sites and mutated them to alanine. Three of the four mutated serine residues were found within the highly conserved SNARE motif of VAMP2. This motif fits to the SNARE motifs of syntaxin-4 and SNAP-23 and would allow a twisted, parallel 4-helical bundle (18). Since the driving forces for the generation of the helical bundle are mostly hydrophobic interactions,

one or several highly polar phosphorylated serine residues could disturb the formation of the bundle. However, several reports showed that decreased binding of SNARE proteins to each other could lead to an increased fusion of vesicles. For example, SNAP-25 is phosphorylated by PKC at S187, which lies within the C-terminal SNARE motif (42-44). Activation of PKC by various agents resulted in phosphorylation of SNAP-25 (43). This phosphorylation decreased binding of SNAP-25 to syntaxin-1 and increased neurotransmitter release, possibly by accelerating the SNARE complex dissociation and thus enhancing the efficiency of exocytosis (43). Similar results have been obtained in the case of PKC-dependent phosphorylation of syntaxin-4 and SNAP-23 (45,46). Thus, PKCζ-mediated phosphorylation of VAMP2 in response to stimulation as shown here may interfere with the interaction of the v-SNARE protein VAMP2 with t-SNARE proteins and may thereby lead to reduced affinity – possibly promoting disassembly and therefore accelerating vesicle trafficking as described for the SNARE proteins above.

Another possibility would be that the phosphorylation of VAMP2 by PKCζ influences its interaction with accessory proteins. Such proteins can up- or downregulate SNARE- to SNARE-protein interactions. For example Munc18c inhibits SNARE- to SNARE-protein interaction and thus negatively regulates insulin-stimulated translocation of GLUT4 to the plasma membrane in adipocytes (47,48). Recently, several interaction partners of VAMP2 such as prenylated Rab acceptor 1 (PRA1) (49), a VAMP2 associated protein of 33 kDa (VAP-33) (50,51), and pantophysin (52) were found in 3T3-L1 cells, however their precise role in vesicle fusion is not yet understood.

Without knowledge of the precise mechanism of action, our results demonstrate that ProF stimulates phosphorylation of the SNARE protein by PKCζ. This could be a possible mechanism by which PKCζ influences vesicle trafficking. Recruitment of PKCζ to VAMP2 by means of ProF enhanced the substrate phosphorylation of the SNARE protein (as depicted in Fig. 5). IGF-1 stimulation increased the phosphorylation of PKCζ and VAMP2. Furthermore, a role of ProF in insulin metabolism can be envisaged. We showed previously in adipocytes that ProF translocates upon insulin- stimulation to the plasma membrane, parallel to GLUT4, and increases glucose uptake (1). At which time point VAMP2 gets phosphorylated by PKCζ we do not know.

The expression profile of ProF suggested a broad tissue distribution. Thus, the ternary complex of ProF, VAMP2 and PKCζ may occur in other tissues as well, possibly involved in other secretory pathways.

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Footnotes

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The abbreviations used are: AS160, Akt substrate of 160 kDa; BSA, Bovine serum albumin; CY-5, cyanine-5; DL, direct lysate; EDTA, ethylenediaminetetraaceticacid; FITC, fluorescein isothiocyanate; FYVE, domain identified in Fab1p, YOTB, VAC1p, and EEA1; GLUT4, glucose transporter type 4; HA, hemagglutinin; HEK, human embryonic kidney cells; IB, immunoblot; IGF-1, insulin-like growth factor: IP, immunoprecipitation; NSF, *N*-ethylmaleimide-sensitive fusion protein; PRA1, Prenylated Rab acceptor 1; PBS, phosphate-buffered saline; PI3P, phosphatidylinositol-3-phosphate; ProF, propeller-FYVE protein; PKA, protein kinase A; PKC, protein kinase C; ProF, propeller FYVE protein; SNAP, synaptosomal-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; TBS, Tris- buffered saline; TRITC, tetramethylrhodamine isothiocyanate; t-SNAREs, target-SNARE;

VAMP2, vesicle-associated membrane protein 2; VAP-33, VAMP associated protein of 33 kDa; v-SNAREs, vesicular-SNARE

Figure legends

Fig.1. VAMP2, a new interaction partner of ProF. *A*, Domain structure of ProF and VAMP2. ProF consists of 7 WD-repeat motifs (WD1-7, indicated as blue boxes) for binding to proteins and a FYVE domain (indicated as black box), which enables binding to PI3P on vesicular membranes (*top panel*). VAMP2 is anchored to vesicular membranes through its C-terminal transmembrane domain (TM). Furthermore, it possesses a central SNARE motif essential for the interaction with its target SNARE proteins on the plasma membrane. Serine residues, which are potential PKCζ-phosphorylation sites are indicated below (*bottom panel*). *B*, Coimmunoprecipitation assay of transiently overexpressed Myc-tagged ProF and Flag-tagged VAMP2 in HEK 293T cells. Immunoprecipitates were analyzed by immunoblotting (IB) with antibodies against Flag- and Myc-epitopes. The upper panel shows the interaction. *C*, Colocalization of Myc-ProF and Flag-VAMP2. Myc-ProF and Flag-VAMP2 were transiently overexpressed in COS-7 cells. Confocal microscopy analysis with Flag-specific and Myc-specific antibodies revealed areas of colocalization as visualized in yellow on the merged picture (*bottom*). *D,* Characterization of the interaction between ProF and VAMP2. Myc-ProF wild-type (wt), ProF lacking the FYVE domain (Myc-ProFΔFYVE), and in addition lacking blades 1-3 (Myc-ProFΔ1-3ΔFYVE) or lacking blades 4-7 (Myc-ProFΔ4-7ΔFYVE) were transiently overexpressed together with Flag-VAMP2 in HEK 293T cells. Interaction of ProF with VAMP2 was analyzed by immunoprecipitation (IP) with an antibody to the Flag-tag followed by immunoblotting (IB) with antibodies against Flag- and Myc-epitopes.

Fig. 2**.** Interaction of ProF and VAMP2 with PKCζ. *A,* Myc-ProF increases the interaction of PKCζ and VAMP2. HA-PKCζ, Flag-VAMP2 and Myc-ProF were transiently overexpressed in HEK 293T cells. Interaction of VAMP2 with ProF and PKCζ was analyzed by immunoprecipitation (IP) with an antibody to the Flag-epitope followed by immunoblotting (IB) with antibodies against HA-, Myc- and Flag-epitopes. *B,* HA-PKCζ, Flag-VAMP2 and Myc-ProF form a ternary complex. HA-PKCζ, Flag-VAMP2 and Myc-ProF were transiently coexpressed in HEK 293T cells. The ternary complex comprising HA-PKCζ, Flag-VAMP2 and Myc-ProF was immunoprecipitated with an anti Flag-antibody. The complex was eluted from the antibody by addition of an excess of a competing Flag-peptide followed by an immunoprecipitation using an antibody directed against the Myc-epitope. Immunoprecipitates of the different steps were analyzed by immunoblotting against HA-, Myc- and Flag-epitope. *Lane 1*, Direct lysate (DL) of HEK 293T cells expressing HA-PKCζ, Flag-VAMP2 and Myc-ProF; *Lane 2*, Complex after immunoprecipitation with anti Flag-antibody; *Lane 3*, Complex after peptide competition with Flag-peptide and subsequent immunoprecipitation with anti-Myc-antibody. *C*, COS-7 cells transiently expressing HA-PKCζ (green), Flag-VAMP2 (red) and Myc-ProF (magenta) were analyzed by confocal microscopy using antibodies against HA-, Flag- and Myc-epitopes. A partial colocalization on cytoplasmic punctuate structures (white) is observed (merged).

Fig. 3. Interaction and colocalization of endogenous PKCζ, VAMP2 and ProF. *A*, Murine brain lysates were treated with an anti-ProF antibody in the presence and absence of an excess of competing peptide used as antigen to raise the anti-ProF antibody. The precipitates were analyzed by immunoblotting for the presence of VAMP2 and ProF. *B*, Immunoprecipitation analysis using antibodies directed against PKCζ, ProF, and VAMP2. Precipitates were immunoblotted against PKCζ, ProF, and VAMP2. *C*, For confocal immunofluorescence analysis of 3T3-L1 pre-adipocytes were serum-starved for 2 hrs prior to staining for endogenous PKCζ/λ (green),

endogenous VAMP2 (red), and Myc-tagged ProF (magenta). A partial colocalization on cytoplasmic punctuate structures (white) is observed (merged and merged, zoomed).

Fig. 4. Phosphorylation of Flag-VAMP2 by PKCζ is increased *in vitro* by ProF. *A*, Flag-VAMP2 was overexpressed in the presence (lane 2 – 3) and absence (lane 1) of HA-PKCζ in HEK 293T cells. Cells were stimulated with 100 ng/ml IGF-1 for 15 min (lanes 1, 3). Flag-VAMP2 and Flag-VAMP2 - HA-PKCζ complexes were immunoprecipitated with an antibody directed against the Flag-epitope. Immunoprecipitates were subjected to a *in vitro* kinase assay (IVKA) with γ-^{32}P-ATP. VAMP2 phosphorylation and PKCζ autophosphorylation were analyzed using a Phosphoimager (panels 1 and 2). Expression of proteins was analyzed by immunoblotting against Flag-epitope (panels 3 and 5) and HA-epitope (panels 4 and 6). The panels 1 – 4 show the immunoprecipitates (IP), panels 5 – 6 the direct lysates (DL). *B*, Flag-VAMP2 wild-type (wt) and VAMP2 serine to alanine mutant (mt(1-4)) were overexpressed in the presence and absence of Myc-ProF in HEK 293T cells. Flag-VAMP2 and Flag-VAMP2 - Myc-ProF complexes were obtained by immunoprecipitation with an antibody directed against the Flag-epitope. Immunoprecipitates were phosphorylated by addition of recombinant PKCζ and γ-^{32}P-ATP. VAMP2 phosphorylation and PKCζ autophosphorylation was analyzed using a Phosphoimager (panel 1 and 2) and expression of proteins by immunoblotting against Flag-epitope (panel 3 and 4) and Myc-epitope (panel 5 and 6).

Fig. 5. Model of the general role of ProF in secretory systems. Stimulation of the cells induces a signal transduction cascade, which leads to activation of a kinase, such as the atypical protein kinase PKCζ. PKCζ is recruited to VAMP2-containing vesicles through the protein ProF. ProF enables the interaction between activated PKCζ and v-SNARE VAMP2, leading to the phosphorylation of VAMP2 by activated PKCζ. The vesicle then translocates to the plasma membrane, where the interaction of VAMP2 with its cognate target t-SNARE induces incorporation of the vesicle membrane into the plasma membrane.

Figure 1

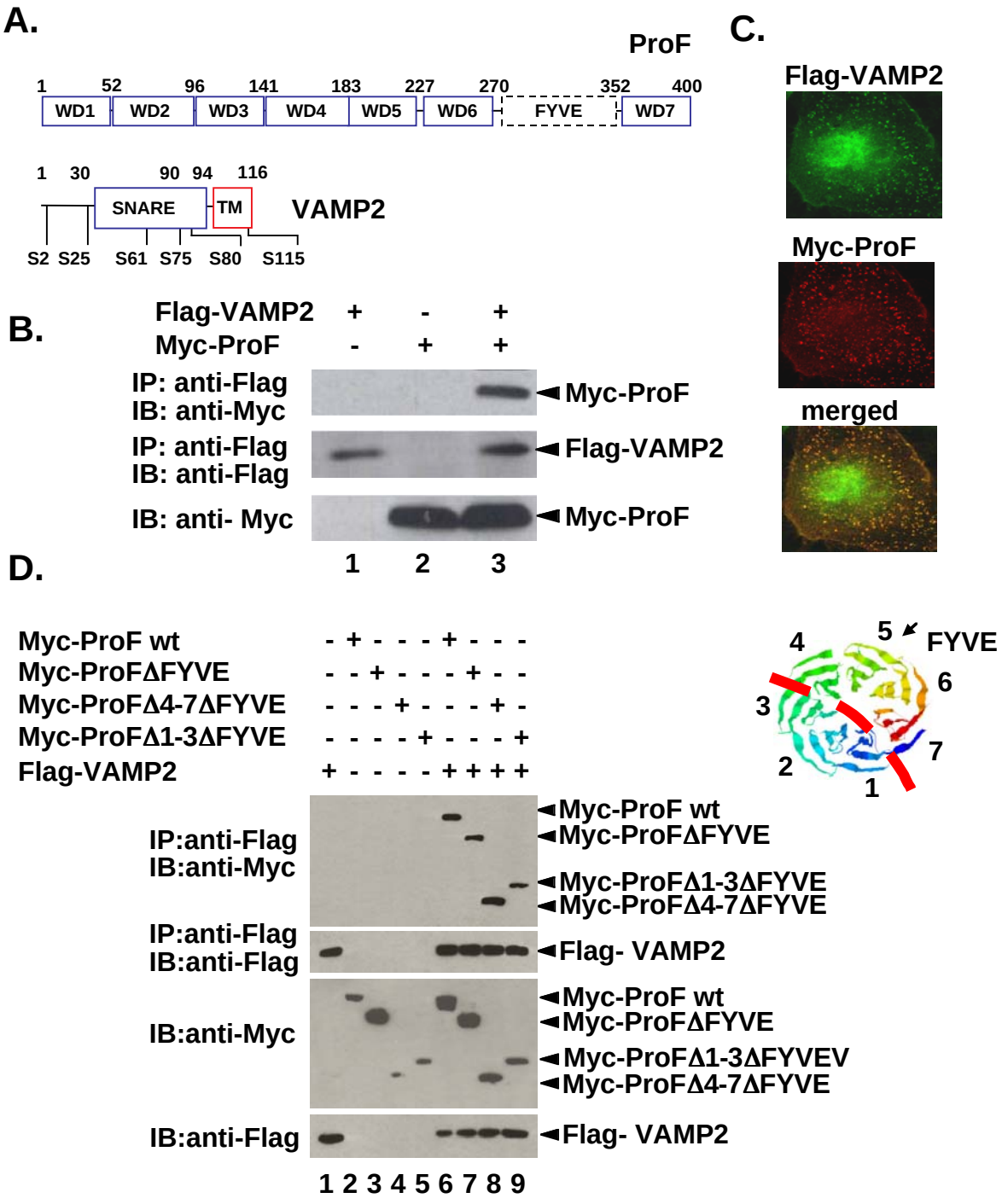
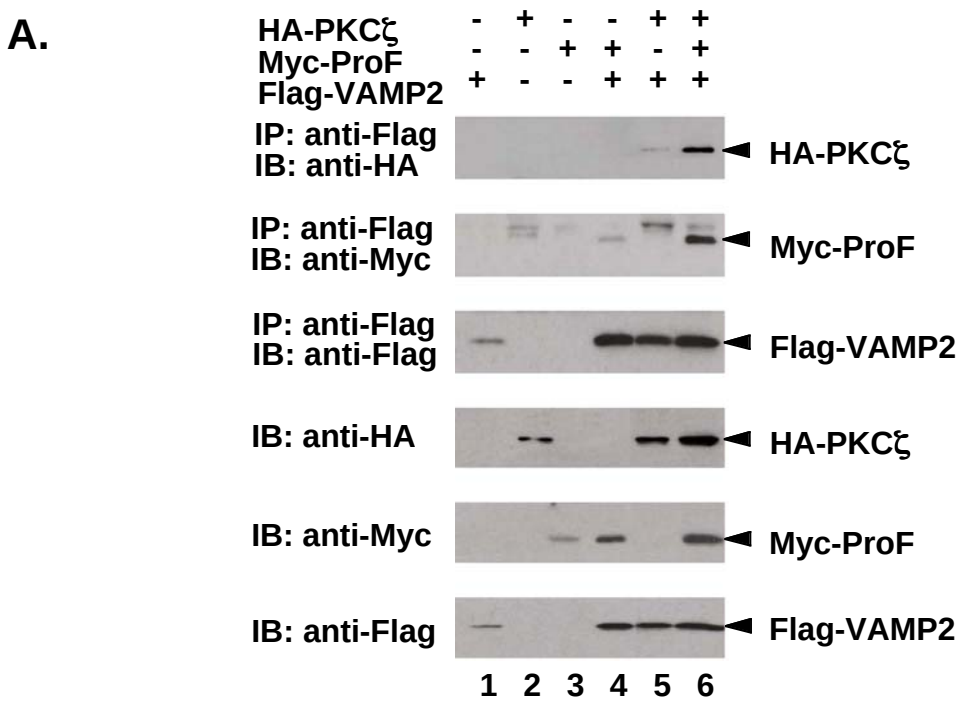
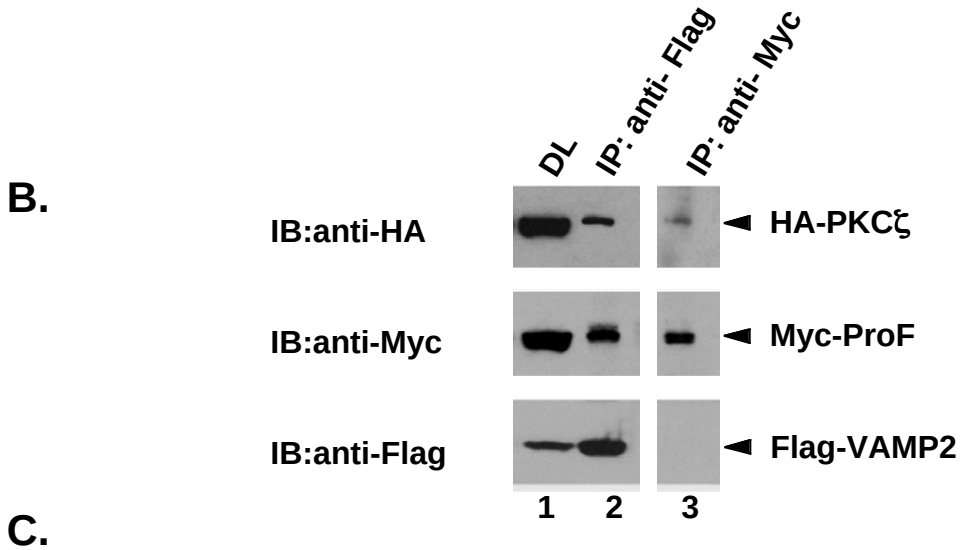

A.
ProF
1 52 96 141 183 227 270 352 400
WD1 WD2 WD3 WD4 WD5 WD6 FYVE WD7
1 30 90 94 116
SNARE TM
VAMP2
S2 S25 S61 S75 S80 S115
B.
Flag-VAMP2 + - +
Myc-ProF - + +
IP: anti-Flag
IB: anti-Myc
Myc-ProF
IP: anti-Flag
IB: anti-Flag
Flag-VAMP2
IB: anti- Myc
Myc-ProF
1 2 3
C.
Flag-VAMP2
Myc-ProF
merged
D.
Myc-ProF wt - + - - - + - - -
Myc-ProFΔFYVE - - + - - - + - -
Myc-ProFΔ4-7ΔFYVE - - - + - - - + -
Myc-ProFΔ1-3ΔFYVE - - - - + - - - +
Flag-VAMP2 + - - - - + + + +
5 FYVE
4
6
3
7
2
1
IP:anti-Flag
IB:anti-Myc
Myc-ProF wt
Myc-ProFΔFYVE
Myc-ProFΔ1-3ΔFYVE
Myc-ProFΔ4-7ΔFYVE
IP:anti-Flag
IB:anti-Flag
Flag- VAMP2
IB:anti-Myc
Myc-ProF wt
Myc-ProFΔFYVE
Myc-ProFΔ1-3ΔFYVEV
Myc-ProFΔ4-7ΔFYVE
IB:anti-Flag
Flag- VAMP2
1 2 3 4 5 6 7 8 9

Figure 2


A.
HA-PKCζ - + - - + +
Myc-ProF - - + + - +
Flag-VAMP2 + - - + + +
IP: anti-Flag
IB: anti-HA
HA-PKCζ
IP: anti-Flag
IB: anti-Myc
Myc-ProF
IP: anti-Flag
IB: anti-Flag
Flag-VAMP2
IB: anti-HA
HA-PKCζ
IB: anti-Myc
Myc-ProF
IB: anti-Flag
Flag-VAMP2
1 2 3 4 5 6


B.
DL
IP: anti- Flag
IP: anti- Myc
IB:anti-HA
HA-PKCζ
IB:anti-Myc
Myc-ProF
IB:anti-Flag
Flag-VAMP2
1 2 3

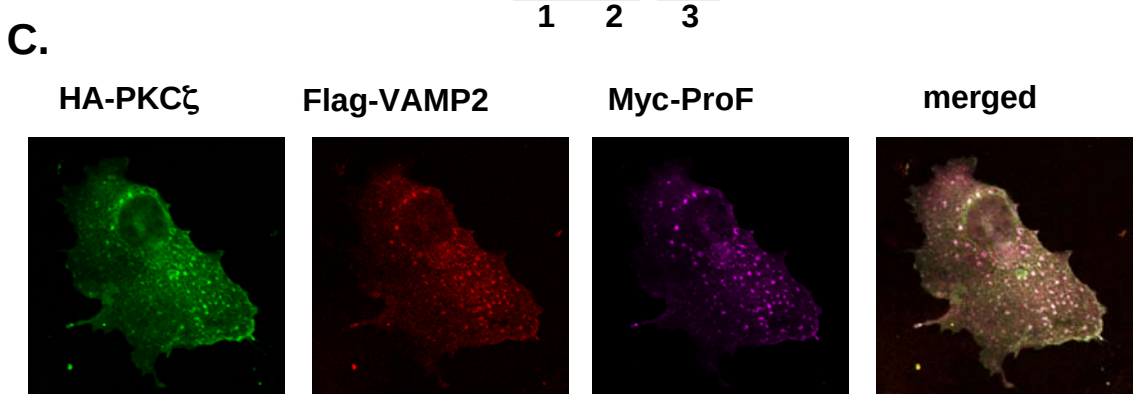

C.
HA-PKCζ
Flag-VAMP2
Myc-ProF
merged

Figure 3

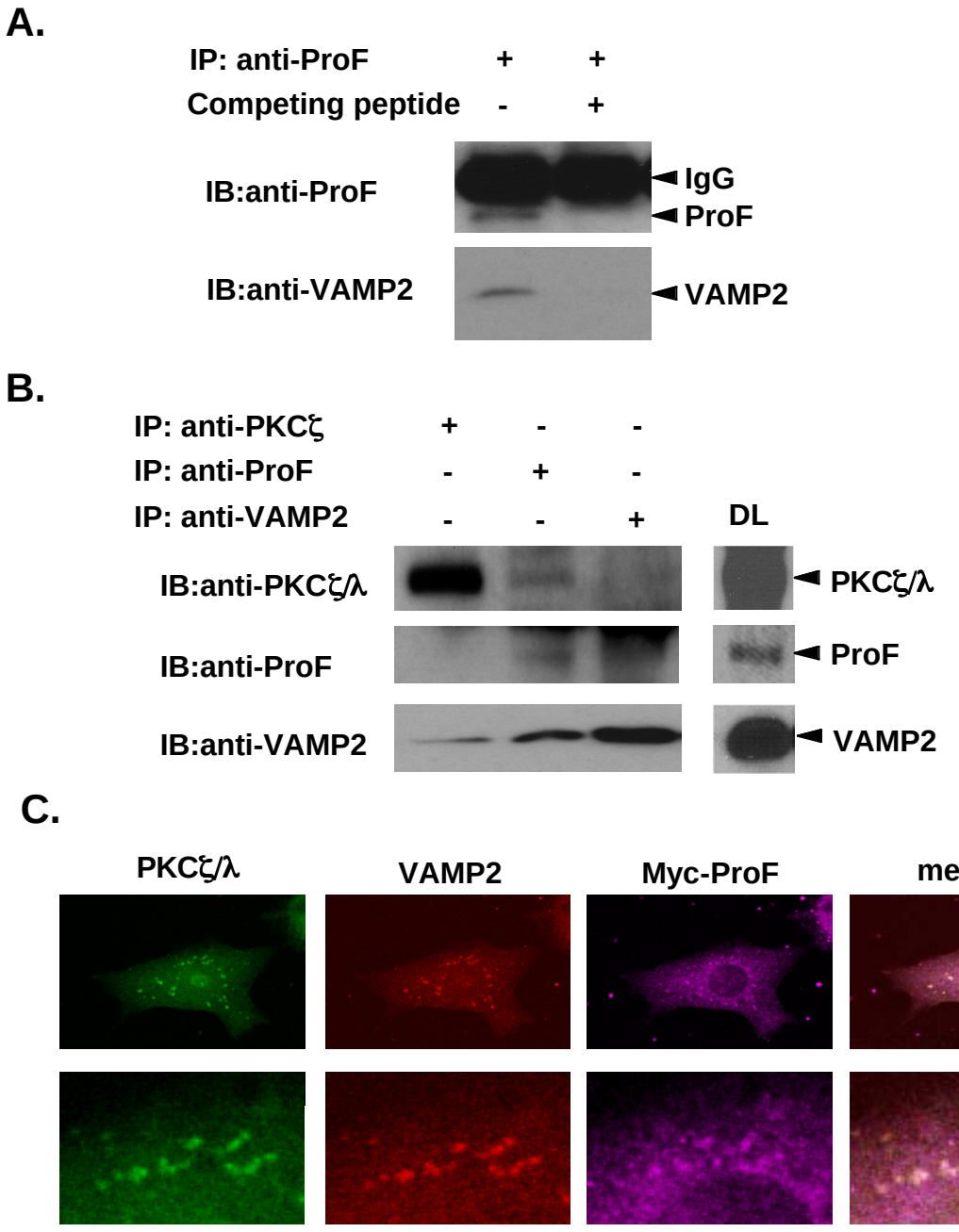
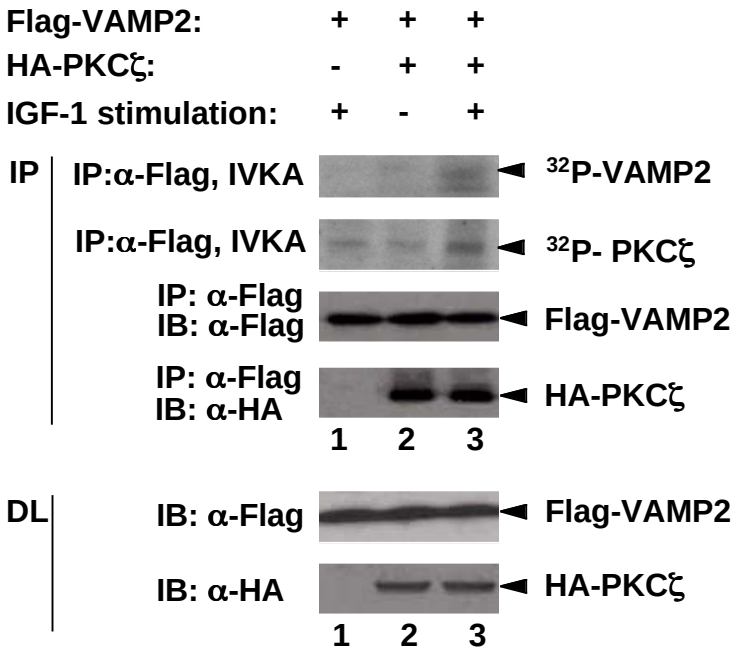

A.
IP: anti-ProF + +
Competing peptide - +
IB:anti-ProF
IgG
ProF
IB:anti-VAMP2
VAMP2
B.
IP: anti-PKCζ + - -
IP: anti-ProF - + -
IP: anti-VAMP2 - - +
DL
IB:anti-PKCζ/λ
PKCζ/λ
IB:anti-ProF
ProF
IB:anti-VAMP2
VAMP2
C.
PKCζ/λ
VAMP2
Myc-ProF
merged

Figure 4

A.


Flag-VAMP2: + + +
HA-PKCζ: - + +
IGF-1 stimulation: + - +
IP
IP:α-Flag, IVKA
32P-VAMP2
IP:α-Flag, IVKA
32P- PKCζ
IP: α-Flag
IB: α-Flag
Flag-VAMP2
IP: α-Flag
IB: α-HA
HA-PKCζ
1 2 3
DL
IB: α-Flag
Flag-VAMP2
IB: α-HA
HA-PKCζ
1 2 3

B.

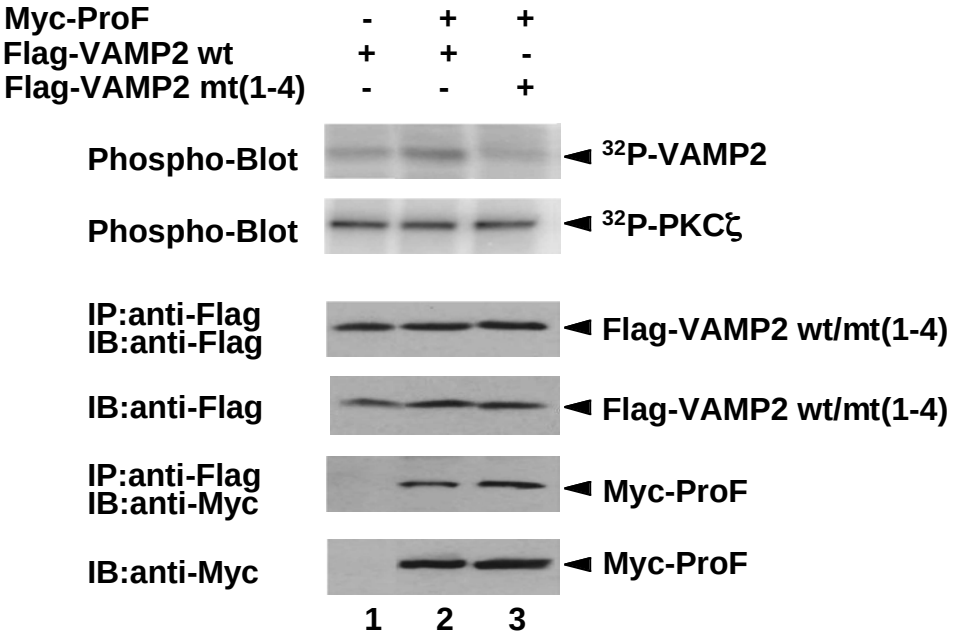
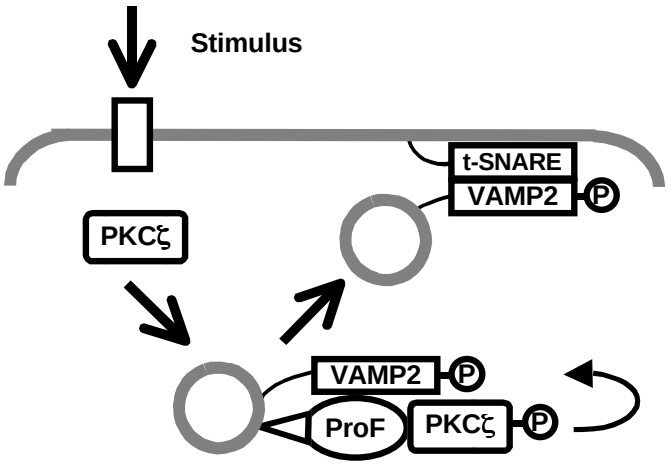

Myc-ProF - + +
Flag-VAMP2 wt + + -
Flag-VAMP2 mt(1-4) - - +
Phospho-Blot
32P-VAMP2
Phospho-Blot
32P-PKCζ
IP:anti-Flag
IB:anti-Flag
Flag-VAMP2 wt/mt(1-4)
IB:anti-Flag
Flag-VAMP2 wt/mt(1-4)
IP:anti-Flag
IB:anti-Myc
Myc-ProF
IB:anti-Myc
Myc-ProF
1 2 3

Figure 5



4.3 WD-FYVE PROTEIN REGULATES DIFFERENTIATION OF PREADIPOCYTES AND GLUCOSE UPTAKE IN ADIPOCYTES

(manuscript to be submitted)

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Running Title: Role of WD-FYVE protein in adipogenesis

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Summary

We have recently identified a protein, consisting of seven WD-repeats, presumably forming a β-propeller, and a FYVE domain, ProF, which binds the activated kinases Akt and protein kinase Cζ/λ (PKCζ/λ) upon stimulation and is involved in glucose uptake in 3T3-L1 adipocytes. Overexpression of ProF led to increased glucose uptake, while its knock down by small interfering RNA (siRNA) caused reduced glucose uptake (1). Here we describe that ProF also influences differentiation of 3T3-L1 preadipocytes into adipocytes. Protein and lipid droplet accumulation was delayed in 3T3-L1 cells with ProF knock down. The effect of downregulation of ProF on protein accumulation and glucose uptake was effective during the onset of differentiation. ProF influenced the expression of several adipocyte differentiation- related proteins, but did not influence the growth rate of the cells. Use of two different siRNAs targeted against the ProF mRNA revealed that complete down regulation of ProF strongly influenced glucose uptake and adipogenesis in a concentration-dependent manner, since partial downregulation had a much weaker effect. Thus, we propose that ProF affects both, early differentiation and glucose uptake of differentiated cells.

Introduction

We have recently identified the propeller- FYVE protein (ProF) as binding partner for Akt and protein kinase Cζ/λ (PKCζ/λ) (1). In adipocytes, ProF translocated together with the kinases and the glucose transporter 4 (GLUT4) to the plasma membrane in response to insulin. As the two kinases play a major role in insulin-dependent GLUT4 translocation from internal vesicles to the plasma membrane in adipocytes (2), we investigated the role of ProF in this process and recently demonstrated that the protein regulates glucose uptake in adipocytes as shown by overexpression as well as downregulation of ProF. In order to better understand the role of ProF in the adipocyte system, we expanded our investigation to the process of adipogenesis, the generation of adipose tissue from fibroblast-like precursor cells. Adipogenesis controls the number of adipocytes present in an organism (3) and strongly increased number and size of adipocytes can result in obesity (4).

Obesity has become a health problem of epidemic proportion both in the developed and the developing world (5). It serves as a

significant risk factor for many diseases such as diabetes type II, cancer, heart disease and hypertension (6-8). Therefore, the investigation of adipogenesis is of high relevance to obesity research.

To address this question, we decided to investigate the role of ProF in the 3T3-L1-cell line, which provides a well-characterized model for the study of adipocyte specific terminal differentiation and adipogenesis (9-12). 3T3-L1 preadipocytes are morphologically similar to fibroblasts and grow exponentially until they reach confluence. This leads to cell-cycle arrest at the G_0/G_1 cell cycle boundary, arrest of proliferation and the expression of early adipocyte differentiation markers, including low density lipoprotein (LDL) (13-15). Exposure of the cells to hormonal agents such as insulin, dexamethasone (DMX) and 3-isobutyl-1-methylxanthine (IBMX) causes the synchronous re-entry of all preadipocytes into the cell cycle by traversing the G_1/S checkpoint, followed by a limited number (approximately two rounds) of mitotic cell divisions. This period is called the mitotic clonal expansion phase (16). DNA synthesis during this period probably alters accessibility of promoter control elements to transcription factors. During this phase intermediate differentiation markers, such as CAATT-enhancer-binding protein (C/EBP)β and C/EBPδ, are expressed, which prime the differentiation pathway (17) by transcriptionally activating the peroxisome proliferator-activated receptor (PPAR)γ and C/EBPα genes (18-21).

The phase of clonal amplification is followed by an unique cell-cycle stage of permanent growth-arrest, called G_D (22,23), which is probably the result of the antimitotic activity of PPARγ and C/EBPα. (24-28). In this phase expression of late differentiation markers occurs. Examples are GLUT4 and the insulin receptor (IR), but also C/EBPα, which is important for maintenance of the terminally differentiated state. Expression of these proteins also leads to downregulation of the extracellular matrix and reorganization of the cytoskeleton (12). Together, this results in differentiation of the cells into mature, fully differentiated adipocytes, as seen by rounding up of cells into a spherical shape and accumulation of large cytosolic lipid vacuoles – the triacylglycerol droplets. After an extended period in culture the vacuoles coalesce and become unilocar, causing the typical signet ring appearance of mature white adipocytes (11).

Adipocyte differentiation is controlled by complex actions involving gene expression and signal transduction. It is mediated by several transcription factors identified as master regulators for adipogenesis. However, the mechanisms underlying the steps, which trigger the initiation of differentiation, remain unknown up to date.

ProF has been shown to be involved in glucose metabolism of 3T3-L1 cells in our previous analysis (1). In the present study we demonstrate that ProF influences both, differentiation of preadipocytes and glucose uptake in mature adipocytes. ProF knock down led to delayed protein and lipid droplet accumulation during the initial phase of differentiation. ProF further influenced the expression of several adipocyte differentiation-related proteins. Use of two different siRNAs targeted against ProF revealed that both, the glucose uptake and the onset of differentiation were dependent on expression levels of ProF.

Results

We have recently identified and characterized a protein, consisting of seven WD-repeats, presumably forming a β-propeller, and a FYVE domain, which we designated as ProF (1). While the WD-repeats of ProF form a protein- protein interaction platform (29), the FYVE domain specifically interacts with phosphatidylinositol-3-phosphate, found on vesicular membranes (30). ProF preferentially bound the activated kinases Akt and PKCζ/λ upon stimulation with insulin-like growth factor 1 (IGF-1). Using adipocytes as model system to understand the role of ProF, we found that the protein translocated with the kinases and glucose transporter 4, GLUT4, to the plasma

membrane in response to insulin stimulation. We also showed that overexpression of ProF led to increased glucose uptake upon insulin stimulation, while knock down of ProF by siRNA led to reduced glucose uptake. This suggested a role of ProF in adipocytes (1).

In order to better understand the function of ProF in this system we analyzed the role of ProF during adipocyte differentiation. To that end, we transduced 3T3-L1 cells with a lentiviral vector expressing a siRNA targeted to ProF mRNA (siProF3), an empty lentiviral vector control (FUGW) or used untransduced (parental) cells. Cells were thereafter subjected to differentiation using a hormonal induction medium (Fig. 1) and monitored by visual inspection using light microscopy. This method is warranted, because the drastic alterations in the morphology of the cell during conversion to spherical shape are highly important for the regulation of adipogenesis. For example, the proteolytic degradation of the extracellular matrix has been reported to promote the expression of critical adipocyte differentiation factors such as C/EBPα and PPARγ (31). Indeed, visual observation of the cells revealed that ProF affects adipocyte differentiation. The differentiation of adipocytes is a multi-step process that successively requires 1.) growth inhibition by cell-cell contact, 2.) hormonal induction, causing mitotic clonal expansion, and 3.) ultimative growth arrest, followed by terminal differentiation – as shown i.e. by rounding up of the cells - and the accumulation of triacylglycerol storage vacuoles – as shown by accumulation of lipid droplets (Fig. 1). While parental and FUGW- transduced 3T3-L1 cells differentiated as expected, we found that siProF3 cells behaved radically different during the phase of mitotic clonal expansion and terminal differentiation. Cells with ProF knock down retained for a longer period a fibroblast-like morphology, and started to round up at least 24 hours later than the control cells (Fig. 2A). However, at the stage of terminal differentiation (day +7) the cells with ProF knock down showed a morphology that was undistinguishable from the appearance of the parental and FUGW- transduced 3T3-L1 cells (Fig. 2A, right). Furthermore, Oil Red O staining of the lipid droplets of the cells demonstrated a delay in lipid accumulation (data not shown).

Because adipocyte differentiation is accompanied by a massive increase in protein content during mitotic clonal expansion (32,33), we decided to investigate protein levels of the cells during adipocyte differentiation by Bradford method. We found that cells transduced with siProF3 showed a clear delay in protein accumulation during mitotic clonal expansion, which is most prominent at day +1. In terminally differentiated cells protein levels of siProF3-expressing cells are indistinguishable from untransduced and control cells. Furthermore, we investigated the cell number of both, untransduced and transduced cells during adipogenesis, because a hallmark of the mitotic clonal expansion phase is a limited number – approximately one to two rounds - of mitotic cell divisions (16). Interestingly, we could not find a difference between siProF3-transduced and untransduced cells, leading us to the assumption that ProF does not influence the clonal expansion as such. In summary, these data show that ProF leads to a delay in both, protein synthesis and lipogenesis, the accumulation of lipid droplets.

Next, we wanted to know whether ProF affected the expression of marker proteins, which regulate adipogenesis. We performed a time course experiment, in which we lysed 3T3-L1 cells at different stages of adipogenesis and subjected the lysates to SDS-PAGE analysis (Fig. 3). First, we investigated the expression levels of ProF. Expression of siProF3 led to the downregulation of ProF3 below detection level, while expression of the empty control vector did not influence ProF expression. Furthermore, we found that expression of the ProF protein increased during hormonal induction, which peaked in the mitotic clonal expansion phase (day –1 to day 0), and gradually declined during terminal differentiation (Figure 3, lane 1). The expression of the PPARγ protein, which is known to be induced at –1 and peaks at day +1 (34), was slightly reduced during mitotic

clonal expansion but was unaffected during terminal differentiation (Figure 3, lane 2). Similarly, the expression of the late differentiation marker GLUT4 was slightly reduced at early time points (day +1), but unaffected during terminal differentiation (Figure 3, lane 4). The expression of Akt2, which is known to be upregulated during adipogenesis (35,36), was slightly affected by ProF knock down (Figure 3, lane 3) at day +0. However, ProF knock down did not alter the expression of the loading control Erk2 (Figure 3, lane 5). These results suggest that ProF affects the expression of a number of marker proteins of adipogenesis.

We further wanted to characterize the role of ProF in adipogenesis by determining the uptake of glucose in siProF3- transduced and control cells. For that purpose, we differentiated 3T3-L1 preadipocytes and measured the uptake of radioactive glucose at different time points during differentiation. At day +1, the insulin-stimulated glucose uptake of siProF3 cells was 50% of the insulin-dependent glucose incorporation of the control cells after standardized for total protein amounts (Fig. 4A and Fig. 4B, left), while the total protein levels were 60% of the control, as determined by Bradford method. At day +4, the insulin-stimulated glucose uptake of siProF3-expressing cells was still only 60% of that obtained for the control cells, whereas the total protein level was already 85% of the control (Fig. 4A and Fig. 4B, middle). At day +7, total protein levels were comparable to those at day +4, but the glucose uptake of siProF3 cells was undistinguishable from that in the untransduced and FUGW-control vector transduced cells (Fig. 4A and Fig. 4B, right). In summary, these data demonstrate that ProF affects glucose uptake in adipocytes, but this effect is limited to the initial phase of differentiation. Furthermore, ProF still influenced glucose uptake at day +4, where total protein levels and cellular morphology (not shown) were almost indistinguishable from the controls.

Next, we wanted to confirm this results by testing if ProF influenced adipogenesis indirectly by increasing the growth or proliferation rate of the cells. If knock down of ProF would slow down the growth of the preadipocyte cells, it would cause a delay of adipogenesis, because adipogenesis is dependent on the confluent state of the cells, which causes cell-cycle arrest at the G_0/G_1 cell cycle boundary, the arrest of proliferation and expression of early adipocyte differentiation markers, including the low density lipoprotein (LDL) (13-15). To test that, 3T3-L1 cells, which were untransduced (parental), transduced with empty vector (FUGW), or transduced with an siRNA-expressing lentiviral vector targeting ProF (siProF3) were seeded for differentiation into adipocytes. In the case of siProF3, cells were seeded in three different concentrations ($6x10^4$ cells, $9x\ 10^4$ cells, and $1{,}2\ x\ 10^5$ cells) to eliminate the possibility of an effect of ProF on cell growth. At day 1 of the differentiation, all cells were subjected to glucose uptake analysis and measured for total protein levels by Bradford method. As can be seen, knock down of ProF led to decreased glucose uptake (Fig. 5A), as well as total decreased protein content (Fig. 5B), and fibroblast-like cell morphology, independent from the initial number of cells. In summary, ProF does not influence glucose uptake in adipocytes by affecting the growth rate of cells, thereby delaying the mitotic clonal expansion.

To gain further insight into the role of ProF in adipocyte differentiation, we introduced a second siRNA against a different sequence of the ProF mRNA. While the siProF3, that was used so far, targets the nucleotides 1.154 – 1.172 of the open reading frame of ProF, the siProF1 is targeted against nucleotides 280 - 300 of the 3'-untranslated region (Fig. 6A). The expression levels of ProF were analyzed by SDS-PAGE analysis. As can be seen, we found that siProF3 led to a downregulation of ProF protein expression below detection levels, whereas siProF1 only caused a partial downregulation of ProF. None of the two siRNAs affected the expression of the tubulin control (Fig. 6B). Next, we compared the influence of ProF knock down on cell morphology and total protein content (Fig. 6C, D). As can be seen, the accumulation

of lipid droplets was markedly delayed in siProF3- transduced cells, but similar to parental cells in siProF1- transduced cells. Also the protein content of siProF1 cells was only slightly decreased in siProF1-, but markedly decreased in siProF3- transduced cells during mitotic clonal expansion, when compared to the untransduced control. Taken together, these data indicate that only complete downregulation of ProF influenced adipogenesis, whereas partial downregulation of ProF3 affected adipogenesis only marginally.

To further strengthen this hypothesis, we performed a glucose uptake with both siProF- cells, compared to untransduced and control- transduced cells (siGL2), in which the siRNA is targeted against firefly luciferase. To better evaluate the role of ProF in adipogenesis, the glucose uptake was measured at two different time points during adipocyte differentiation - day +1(Fig. 7, top) and day +4 (Fig. 7, bottom). As can be seen, glucose uptake was insignificantly decreased in siProF1 cells (35% at day +1, and 30% at day +4), but significantly decreased in siProF3 cells, both at day +1 (70% in comparison to untransduced control) and day +4 (50% in comparison to untransduced control) of adipocyte differentiation (Fig. 7A, B). Interestingly, analysis of the cell morphology (Fig. 7C) and the total protein content (Fig. 7D) revealed that siProF3 influences both markers for adipogenesis noticeably at day +1, but only slightly at day +4, while siProF1 showed only little effect on adipocyte differentiation at both time points. In summary, these data show that both, reduced glucose uptake and delayed adipogenesis, are dependent on ProF- protein levels, since a more efficient knock down led to decreased glucose uptake and delayed adipogenesis. The results furthermore demonstrate that ProF affects glucose uptake independently from its influence on adipogenesis, since glucose uptake was still markedly reduced at day +4, when cells were morphologically indistinguishable from siGL2 and untransduced control cells.

Discussion

We have previously identified ProF as an adaptor molecule, which regulates glucose uptake in adipocytes (1). Overexpression of ProF led to increased glucose uptake, while knock down of ProF by siRNA led to reduced glucose uptake. This raised the question whether ProF might also influence adipogenesis, the differentiation of preadipocytes into adipocytes (1). We addressed this question by using different experimental approaches. The effect of ProF on adipogenesis was determined by observation of the morphology, determination of protein content, cell number, expression of marker proteins by measuring the glucose uptake. For our experiments we used the murine 3T3-L1 cell line as an established and reliable model for studying adipocyte development including growth, metabolism and differentiation. (8,37).

We found that ProF influences differentiation of 3T3-L1 preadipocytes into adipocytes. Protein and lipid droplet accumulation was delayed in 3T3-L1 cells with ProF knock down. The effect of ProF- knock down on protein accumulation and glucose uptake was limited to the first days of differentiation. ProF did not influence the growth rate of the cells, but influenced the expression of several adipocyte differentiation- related proteins. Use of two different siRNAs targeted against ProF revealed that complete downregulation of ProF strongly influenced glucose uptake and adipogenesis, whereas partial downregulation had much weaker effects. Based on these results we conclude that ProF affects both, glucose uptake in adipocytes and adipogenesis of preadipocytes.

The clarification of how ProF is able to influence both processes will be of great interest in future studies. We recently found that ProF binds activated Akt upon growth factor- stimulation and that insulin stimulation leads to a localization of both, ProF and Akt, at the plasma membrane of adipocytes (1).

Insulin is well known to play a critical role in adipocyte differentiation (38-41) and the importance of insulin and Akt for adipogenesis

is supported by several Akt-, phosphatidyl inositol-3-kinase-, and insulin receptor- knock out mice (39-47). Furthermore, overexpression of a constitutively active form of Akt induces adipocyte differentiation (48). However, the molecular pathways downstream of Akt are not clearly understood (48,49). One could envisage that ProF acts as an interaction partner of Akt and, thus, could be involved in bringing together the kinase with a substrate molecule, involved in adipocyte differentiation.

In the last years, a number of Akt targets, which play a role in this process, have been uncovered. One target of Akt, implicated in adipogenesis is the protein mammalian target of rapamycin (mTOR), a serine/threonine kinase (50,51), which is highly upregulated during adipocyte differentiation (52). mTOR phosphorylation causes changes in the phosphorylation of p70 ribosomal protein S6 kinase ($p70^{S6K}$) and eukaryotic initiation factor 4E binding protein 1, (eIF4E-BP1) (53).

Phosphorylation of $p70^{S6K}$ leads for example to phosphorylation of the cAMP response element binding protein (CREB), a transcription factor that promotes adipogenesis (54,55), while eIF4E-BP1 phosphorylation allows activation of protein translation (56). The immunosuppressant drug rapamycin, which inhibits the function of mTOR (53,57-59), represses adipocyte differentiation and lipogenesis in 3T3-L1 cells (60-62). Interestingly, mTOR is found predominantly on internal vesicles of mammalian cells (52). We have recently found that in unstimulated adipocytes ProF is also located at internal vesicles (1).

Another target of Akt are the Foxo transcription factors (63). Phosphorylation by Akt causes inhibition of their transcriptional activity (64,65). Foxo1, the most abundant Foxo isoform in adipocytes is induced in the early stages of adipocyte differentiation, but its activation is delayed by Akt phosphorylation until the end of the clonal expansion phase (66). Akt phosphorylation of other transcription factors, such as GATA2, has also recently been found to promote adipocyte differentiation (67).

More recently, Akt activity was found to be required to induce expression of the key regulator of adipogenesis PPARγ, because Akt1/2 double knock-out resulted in impaired PPARγ induction (68). Interestingly, we found that knock down of ProF leads to delayed expression of PPARγ during mitotic clonal expansion phase (Fig. 3). Furthermore, we have shown earlier that ProF is able to interact with both isoforms, Akt1 as well as Akt2 (1). It will also be interesting to see in further experiments if ProF downregulation by siRNA influences C/EBPβ and C/EBPδ expression, as this has not been found in Akt1/2 double knock out mice.

Taken together, our data support the idea, that ProF could be a positive regulator of both, adipogenesis and insulin-dependent glucose uptake in adipocytes. It is worth noting, that rapamycin, a negative regulator of adipocyte differentiation has been proposed to suppress both, adipogenesis in preadipocytes as well as glucose uptake – and other insulin actions - in fully differentiated adipocytes (69). ProF could have the opposite effect on adipocytes.

Experimental procedures

Reagents and antibodies - Cell culture media, supplements and Novex® 8–16% and 10–20% Tris- Glycine gradient gels were purchased from Invitrogen. Insulin was obtained from Novo Nordisk. Isobutylmethylxanthin (IBMX) and dexamethason were obtained from Sigma. The transfection reagent Lipofectamin 2000 was purchased from Invitrogen. Protease inhibitors were from Roche, 2-Deoxy- D-(1-^{3}H)-glucose, and D-(U-^{14}C)-glucose were obtained from Amersham Pharmacia Biotech. Rabbit polyclonal (C-14) against Erk2, mouse monoclonal (B-7) against tubulin, rabbit polyclonal (H-100) against PPARγ, and goat polyclonal C-20 against GLUT4, were obtained from Santa Cruz Biotechnology, sheep polyclonal against Akt2 from Upstate Biotechnologies.

ProF antiserum - An anti-ProF antiserum was raised in rabbits against a peptide corresponding to the 15 amino acids at the C-

terminus of murine and human ProF. Some of the antiserum was thereafter affinity-purified to increase the specificity of binding (Eurogentech Belgium). Competition experiments were performed with the same peptide to verify the specificity (data not shown).

Cell culture - The murine fibroblastic cell line 3T3-L1 (ATCC number CL-173) was grown in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal calf serum (FCS; Seratec). Penicillin and Streptomycin were added to cultures of 3T3-L1 cells.

Differentiation of adipocytes - For differentiation, early passage 3T3-L1 fibroblasts, were grown in growth medium (DMEM supplemented with 10% FCS) to confluency followed by medium change. 72 h later differentiation was induced by growth medium supplemented with 166 nM insulin, 0.1 µg/ml dexamethason, and 112 µg/ml IBMX. Daily replacement of the hormonal differentiation medium was performed for 3 days. Then, at day 0, the medium was replaced by growth medium containing 166 nM insulin. For insulin-stimulation experiments the cells were starved in DMEM containing 0.5% FCS for 20 h and stimulated for 1 h as indicated. Cells were used for experiments within 20 days after removal of the hormonal differentiation medium. Differentiation was monitored by visual inspection or Oil Red O staining (not shown).

Retroviral transduction and generation of stably transduced 3T3-L1 cells - Retroviruses containing the construct pRTP-Myc-ProF or the empty pRTP vector as control were produced using the BOSC-23 packaging cell-line as described (70,71). Early passage 3T3-L1 fibroblasts were incubated in virus-containing medium for 48 h in the presence of tetracycline (40 ng/ml) to suppress ProF expression. The cells were used in the absence of tetracycline for immunofluorescence studies.
For siRNA downregulation the sequence used was nt 280 - 300 of ProF 3'-UTR, (5'- CCA CTG TTA CCG CAA TCT A - 3') for siProF1, and nt 1154 – 1172 of ProF open reading frame, targeted against exon 11 (5'- GAA CTG ACA AGG TAA TTA A- 3') for siProF3. A 64-nt oligonucleotide, containing the target both in sense and antisense orientation was cloned into pSUPER and then together with the H1 promoter into the lentiviral vector FUGW (72). 4 x 10^5 HEK293T cells were transfected with 0.6 µg of the lentiviral expression construct, 0.6 µg of HCMV-G and 0.6 µg pCMVDR8.3 helper virus plasmids using Lipofectamine 2000 (Invitrogen), followed by medium change after 24 h. The supernatant was used to infect 8 x 10^4 cells of low passage 3T3-L1 cells.

Immunoprecipitation and Western blot analysis - 3T3-L1 cells were extracted in NETN- buffer containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% Nonidet NP-40, 1 mM EDTA using a tight-fitting tissue blender. Complete homogenization was achieved after 6-10 strokes, 10 s each. Lysates were solubilized by shaking for 30 min at 4 °C. The endogenous ProF protein was detected as 44 kD form in direct lysates and precipitates. A 40 kD protein, also competed by the peptide, was observed in some cases with cultured cells and is likely to be unspecific.

Glucose uptake measurements - For Figure 4 and 5, differentiated adipocytes were starved for 20 h in DMEM containing 0.5% FCS, and stimulated for 1 h with insulin in the presence of 1ml of 0.4 µCi/ml D-(U-^{14}C)-glucose (Amersham) with a specific activity of 311 mCi/mmol. Cells were lysed in radioimmunoprecipitation assay buffer (73) and lysates were subjected to scintillation counting analysis for measurement of ^{14}C-glucose uptake.
For Figure 6, differentiated adipocytes were starved for 20 h in DMEM containing 0.5% FCS, and glucose uptake analysis was performed as described in (74) with slight modifications. Briefly, cells were incubated for 30 min at 37°C with 1 mL of DMEM containing 100 nM insulin, washed three-times with 2 ml of warm HEPES- buffered Krebs-

Ringer phosphate buffer (120 mM NaCl, 5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES, pH 7.2) and incubated for 10 min in the same buffer in the presence of 1ml of 1 µCi/ml 2-Deoxy-D-(1-^{3}H)-glucose (Amersham) with a specific activity of 0.8 Ci/mmol. Cells were lysed in radioimmunoprecipitation assay buffer (73) and lysates were subjected to scintillation counting analysis for measurement of ^{3}H-glucose uptake.

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Figure legends

Figure 1: Timeline of adipocyte differentiation. The major hallmarks in adipogenesis, the process of preadipocyte differentiation into adipocytes, are schematically indicated by arrows (top). Day + 0 in the time line corresponds to the day of removal of the hormonal induction medium (indicated by a red bar). The pictures below show the general morphology of the 3T3-L1 cells during cell cycle arrest, hormonal induction, growth arrest and terminal differentiation.

Figure 2: Role of ProF on glucose uptake during adipocyte differentiation. A, Levels of protein expression were analyzed in untransduced, parental (par.), empty vector transduced (FUGW) and cells transduced with an siRNA-expressing lentiviral vector targeting ProF (siProF). Levels of ProF protein expression were analyzed by IB (top lane), total protein levels were standardized to Erk2 (bottom lane). B, (top, left) 3T3-L1 cells, which were untransduced (parental), transduced with empty vector (FUGW), or transduced by a siRNA targeted against ProF (siProF3) were analyzed by light microscopy during different time points of adipocyte differentiation. Day + 0 corresponds to the day of

removal of the hormonal induction medium. (right) The number of lipid droplets in randomly selected areas during adipogenesis is depicted (right). The accumulation of protein during adipogenesis was analyzed by Bradford method (bottom). C, the number of living cells during different time points of adipogenesis was analyzed by Trypan Blue staining.

Figure 3: ProF influences expression of adipogenesis- related genes. 3T3-L1 cells, which were untransduced (p), transduced with empty vector (F), or transduced by a siRNA-expressing lentiviral vector targeting ProF (si) were lysed at different time points of adipocytes differentiation. Time-course analysis of expression of adipocyte differentiation markers was performed by Western blot with antibodies against ProF, PPARγ, Akt2, GLUT4, and Erk2 (from top to bottom).

Figure 4: Effect of ProF knock down is limited to the first days of adipocyte differentiation. A, (top) 3T3-L1 cells, which were untransduced (p), transduced with FUGW empty vector (F), or transduced by a siRNA-expressing lentiviral vector targeting ProF (si) were subjected to glucose uptake analysis during different time points of adipocyte differentiation. For that, cells were starved for 20 h and stimulated for 1 h with insulin in the presence of ^{14}C-glucose (filled column) or left unstimulated (open column). Data are mean values ± SD of 3 points. Asterisk indicate statistically significant difference in glucose uptake (Student's t-test, ** = $P \leq 0.01$). B, Levels of total protein expression during adipocyte differentiation were analyzed by Bradford method (bottom).

Figure 5: ProF does not influence glucose uptake in adipocytes by affecting the growth rate of cells. A, 3T3-L1 cells, which were untransduced (parental), transduced with empty vector (FUGW), or transduced by a siRNA-expressing lentiviral vector targeting ProF (siProF3) were seeded for differentiation into adipocytes. In the case of siProF3 cells were seeded in three different concentrations ($6x10^4$ cells, $9x\ 10^4$ cells, and $1{,}2\ x\ 10^5$ cells) to eliminate the effect of ProF on cell growth. At day 1 of the differentiation, all cells were subjected to glucose uptake analysis (top). B, C. Simultaneously total protein levels of the cell were determined by Bradford method (B) and morphology of the cells was analyzed by light microscopy (C).

Figure 6: Use of different siRNA to evaluate the role of ProF. A, Levels of ProF expression were analyzed by IB (top lane), total protein levels were standardized to tubulin (bottom lane). 1 is direct lysate from untransduced cells, 2 is direct lysate from siGL2 transduced cells, 3 is siProF1-expressing cells, 4 is siProF3-expressing cells. B, 3T3-L1 cells, which were untransduced (parental), or transduced with siRNAs targeted against ProF (siProF1 and siProF3) were analyzed by light microscopy during different time points of adipocyte differentiation. Day + 0 corresponds to the day of removal of the hormonal induction medium. C, Accumulation of protein during adipogenesis was analyzed by Bradford method.

Figure 7: ProF influences glucose uptake and adipogenesis. A, B, 3T3-L1 cells, which were untransduced (parental), transduced with a control siRNA against firefly luciferase (siGL2), or transduced by siRNAs targeted against ProF (siProF1 and siProF3) were subjected to glucose uptake analysis at day +1 of adipocyte differentiation (A) or day +4 of adipocyte differentiation (B). For that, cells were starved for 20 h and stimulated for 30 min with insulin (filled column) or left unstimulated (open column). Afterwards cells were incubated with 2-Deoxy-D-(1-^{3}H)-glucose. Data are mean values ± SD of 3 points. Asterisk indicate statistically significant difference in glucose uptake (Student's t-test, * = $P \leq 0.05$, ** = $P \leq 0.01$). C, D. Morphology of the cells was analyzed by light microscopy (C), while total protein content at day +1 (left) and day +4 (right) was determined by Bradford method (D). For (D), 1 is untransduced cells, 2 is siGL2 transduced cells, 3 is siProF1-expressing cells, 4 is siProF3-expressing cells.

Figure 8: Model for the role of Akt and ProF in adipogenesis. Stimulation of cells by glucocorticoids such as IBMX and DMX leads to elevated cAMP levels and expression of transcription factors, while insulin triggers a signal transduction cascade, which causes activation of Akt. Akt itself, targets several proteins, implicated in adipogenesis such as mTOR (bottom) and Foxo (right). ProF, as adaptor protein that binds Akt and potentially recruits Akt substrates, might influence glucose uptake in such a way.

Figure 1

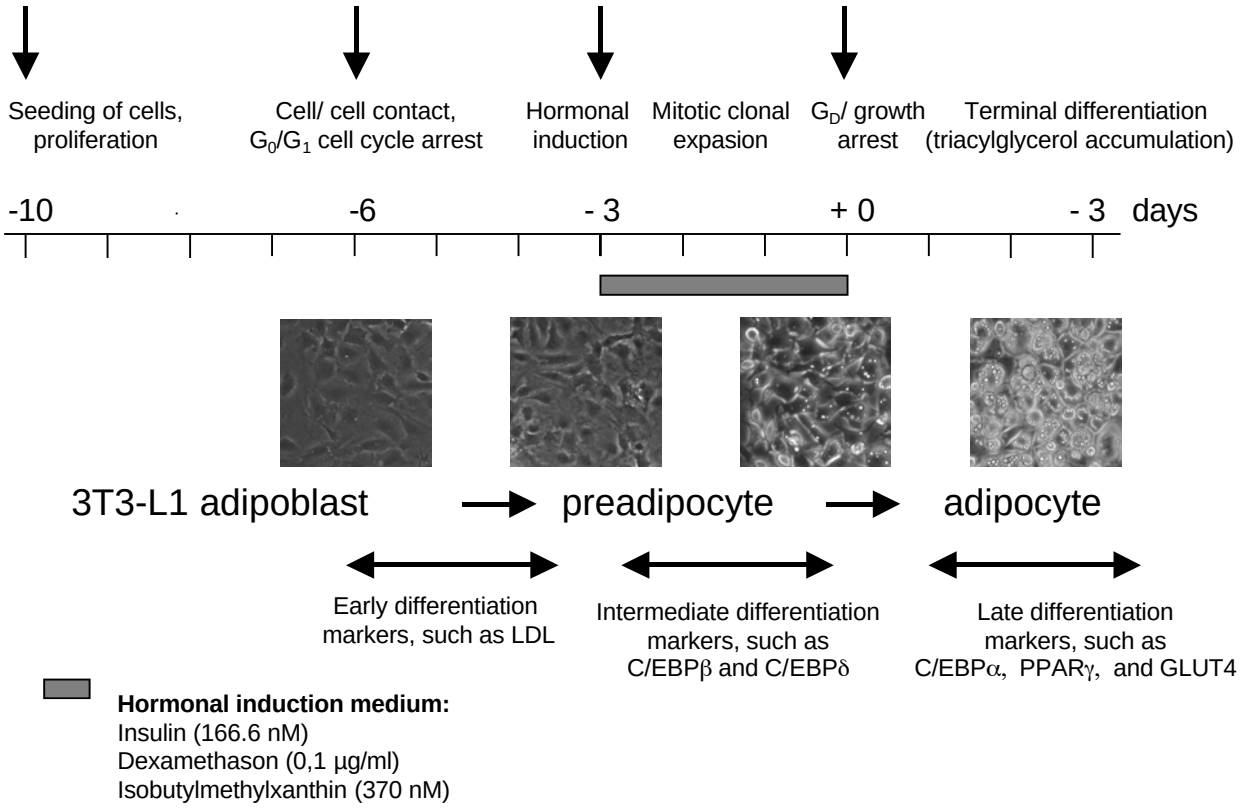

Seeding of cells, proliferation
Cell/ cell contact, G_0/G_1 cell cycle arrest
Hormonal induction
Mitotic clonal expasion
G_D/ growth arrest
Terminal differentiation (triacylglycerol accumulation)
-10
-6
- 3
+ 0
- 3 days
3T3-L1 adipoblast
preadipocyte
adipocyte
Early differentiation markers, such as LDL
Intermediate differentiation markers, such as C/EBPβ and C/EBPδ
Late differentiation markers, such as C/EBPα, PPARγ, and GLUT4
Hormonal induction medium:
Insulin (166.6 nM)
Dexamethason (0,1 µg/ml)
Isobutylmethylxanthin (370 nM)

Figure 2

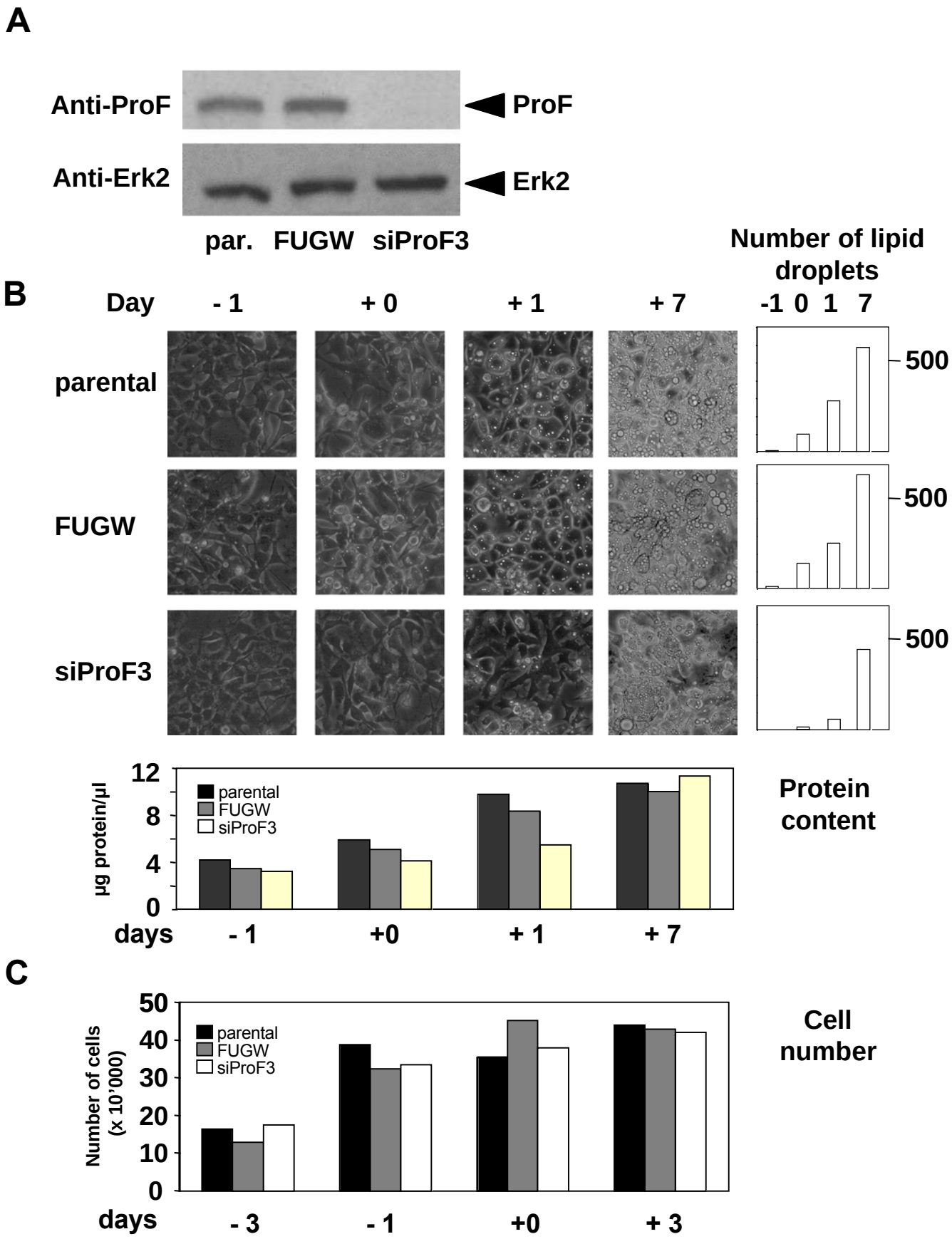

A
Anti-ProF
ProF
Anti-Erk2
Erk2
par.
FUGW
siProF3
B
Day
- 1
+ 0
+ 1
+ 7
Number of lipid droplets
-1 0 1 7
parental
FUGW
siProF3
500
500
500
µg protein/µl
12
8
4
0
parental
FUGW
siProF3
days
- 1
+0
+ 1
+ 7
Protein content
C
Number of cells (x 10'000)
50
40
30
20
10
0
parental
FUGW
siProF3
days
- 3
- 1
+0
+ 3
Cell number

Figure 3

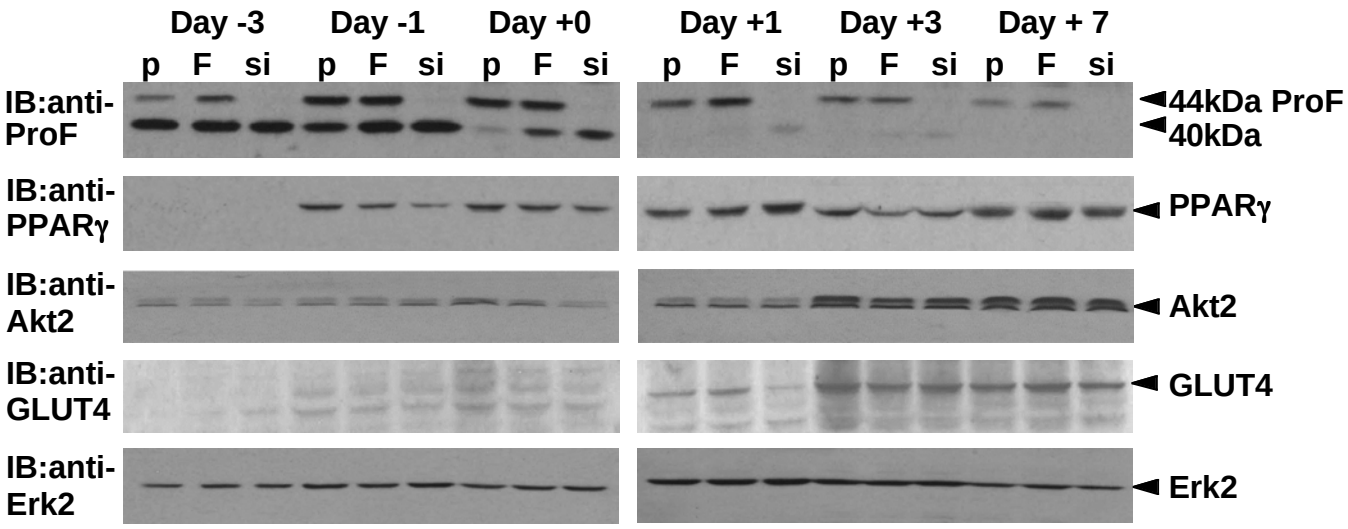
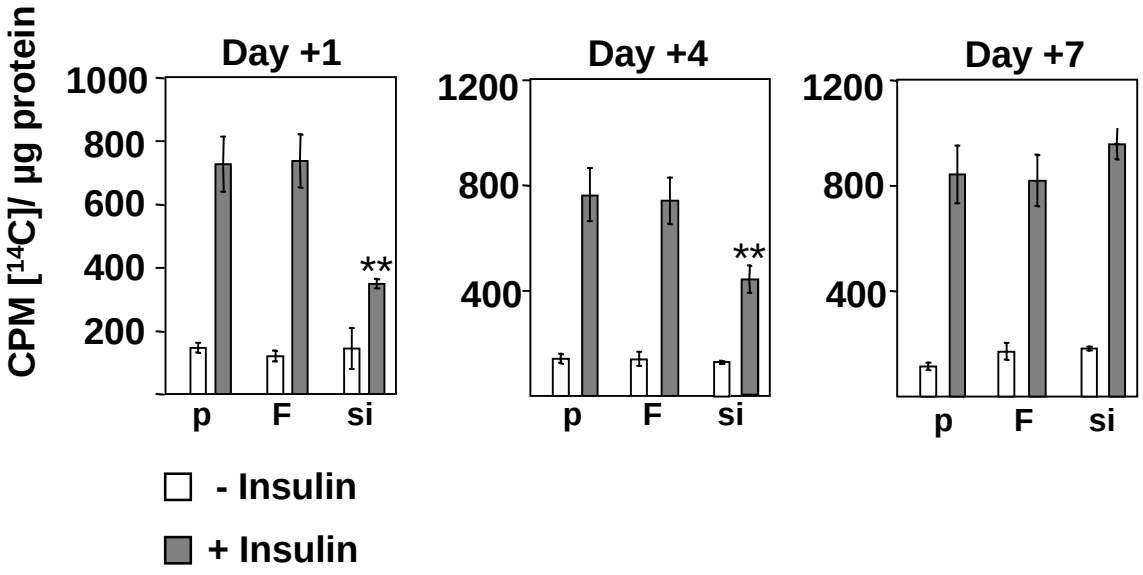

Day -3
Day -1
Day +0
Day +1
Day +3
Day + 7
p F si p F si p F si p F si p F si p F si
IB:anti-ProF
44kDa ProF
40kDa
IB:anti-PPARγ
PPARγ
IB:anti-Akt2
Akt2
IB:anti-GLUT4
GLUT4
IB:anti-Erk2
Erk2

Figure 4

A


CPM [14C]/ µg protein
Day +1
Day +4
Day +7
1000
800
600
400
200
1200
800
400
1200
800
400
**
**
p
F
si
p
F
si
p
F
si
- Insulin
+ Insulin

B

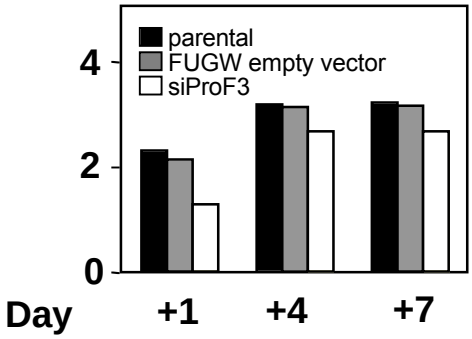

parental
FUGW empty vector
siProF3
4
2
0
Day
+1
+4
+7

Figure 5

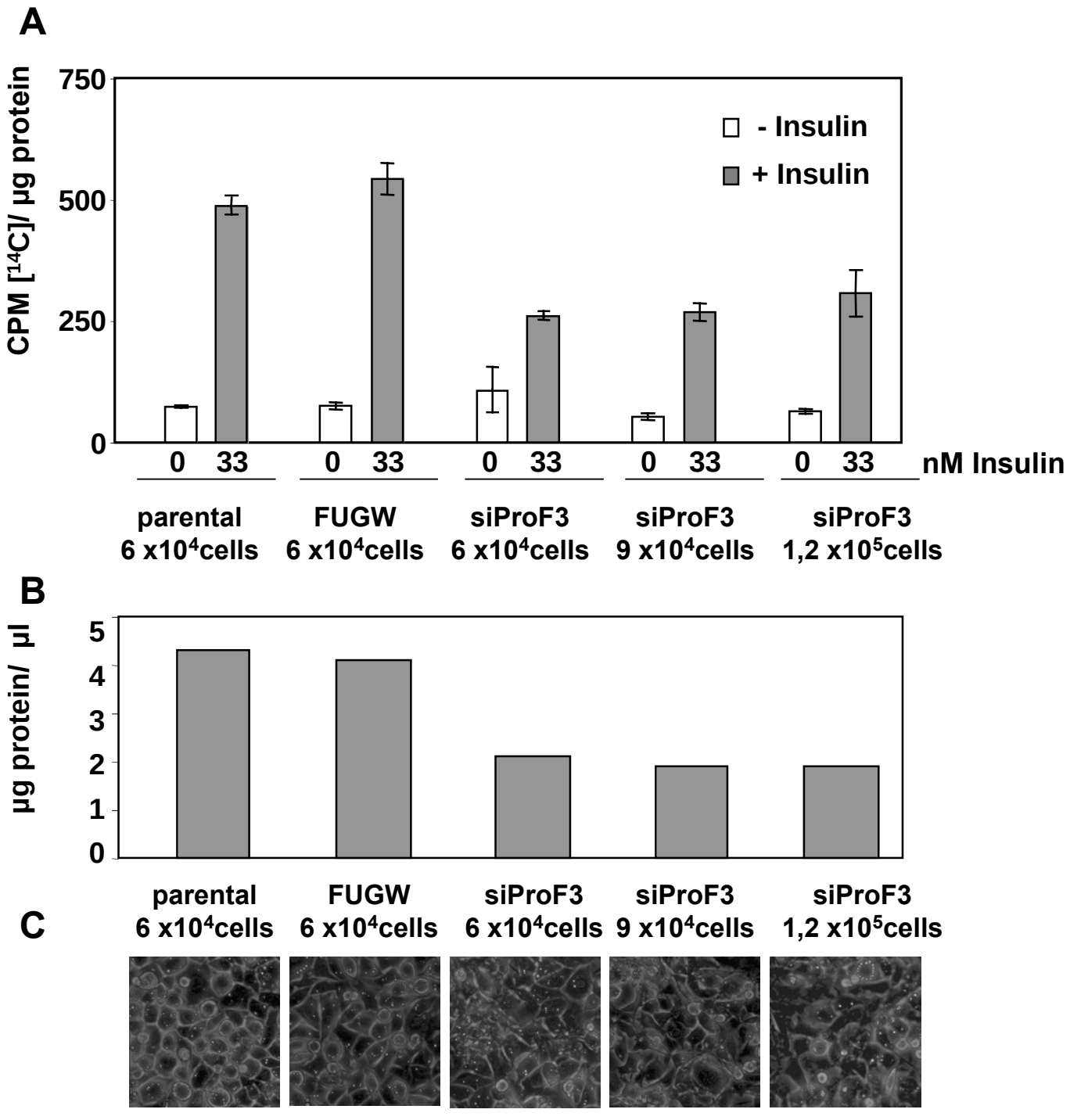
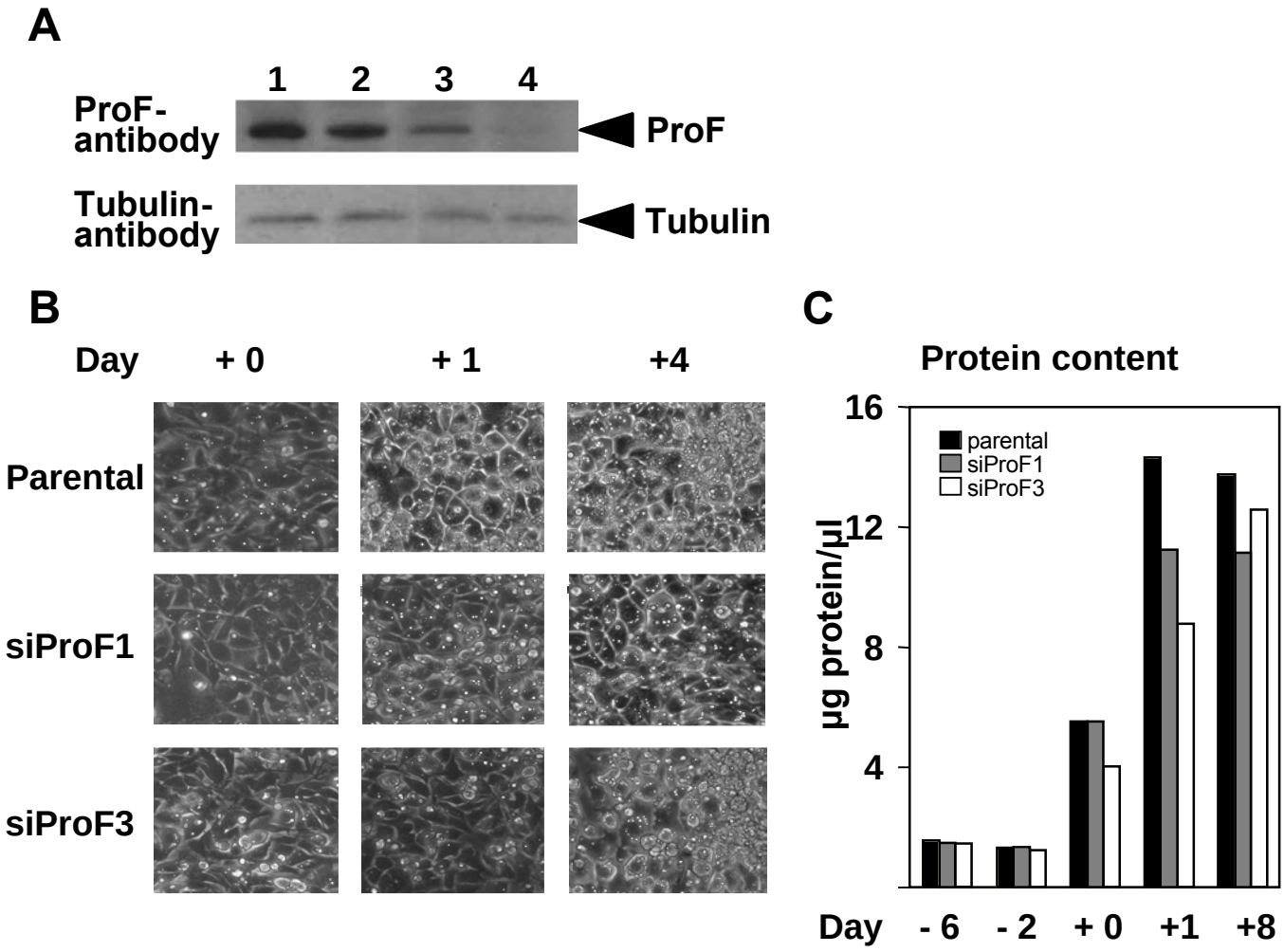

A
CPM [14C]/ µg protein
750
500
250
0
- Insulin
+ Insulin
0 33 0 33 0 33 0 33 0 33
nM Insulin
parental 6 x10⁴cells
FUGW 6 x10⁴cells
siProF3 6 x10⁴cells
siProF3 9 x10⁴cells
siProF3 1,2 x10⁵cells
B
µg protein/ µl
5
4
3
2
1
0
parental 6 x10⁴cells
FUGW 6 x10⁴cells
siProF3 6 x10⁴cells
siProF3 9 x10⁴cells
siProF3 1,2 x10⁵cells
C

Figure 6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1
2
3
4
ProF-antibody
ProF
Tubulin-antibody
Tubulin
B
Day
+ 0
+ 1
+4
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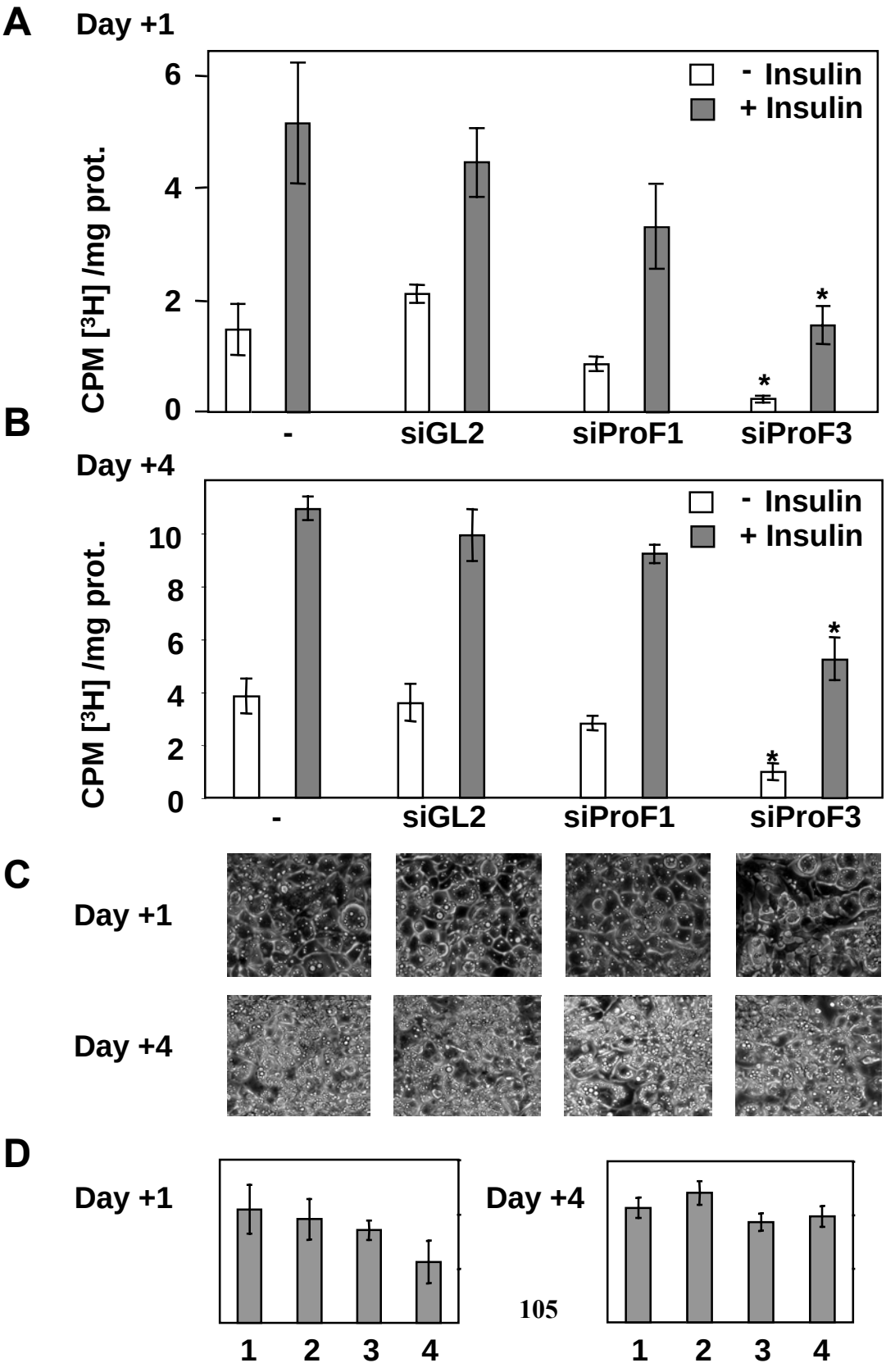
siProF3
C
Protein content
16
12
8
4
µg protein/µl
parental
siProF1
siProF3
Day
- 6
- 2
+ 0
+1
+8

Figure 7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Day +1
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6
4
2
0
- Insulin
+ Insulin
*
*
-
siGL2
siProF1
siProF3
B
Day +4
CPM [3H] /mg prot.
10
8
6
4
2
0
- Insulin
+ Insulin
*
*
-
siGL2
siProF1
siProF3
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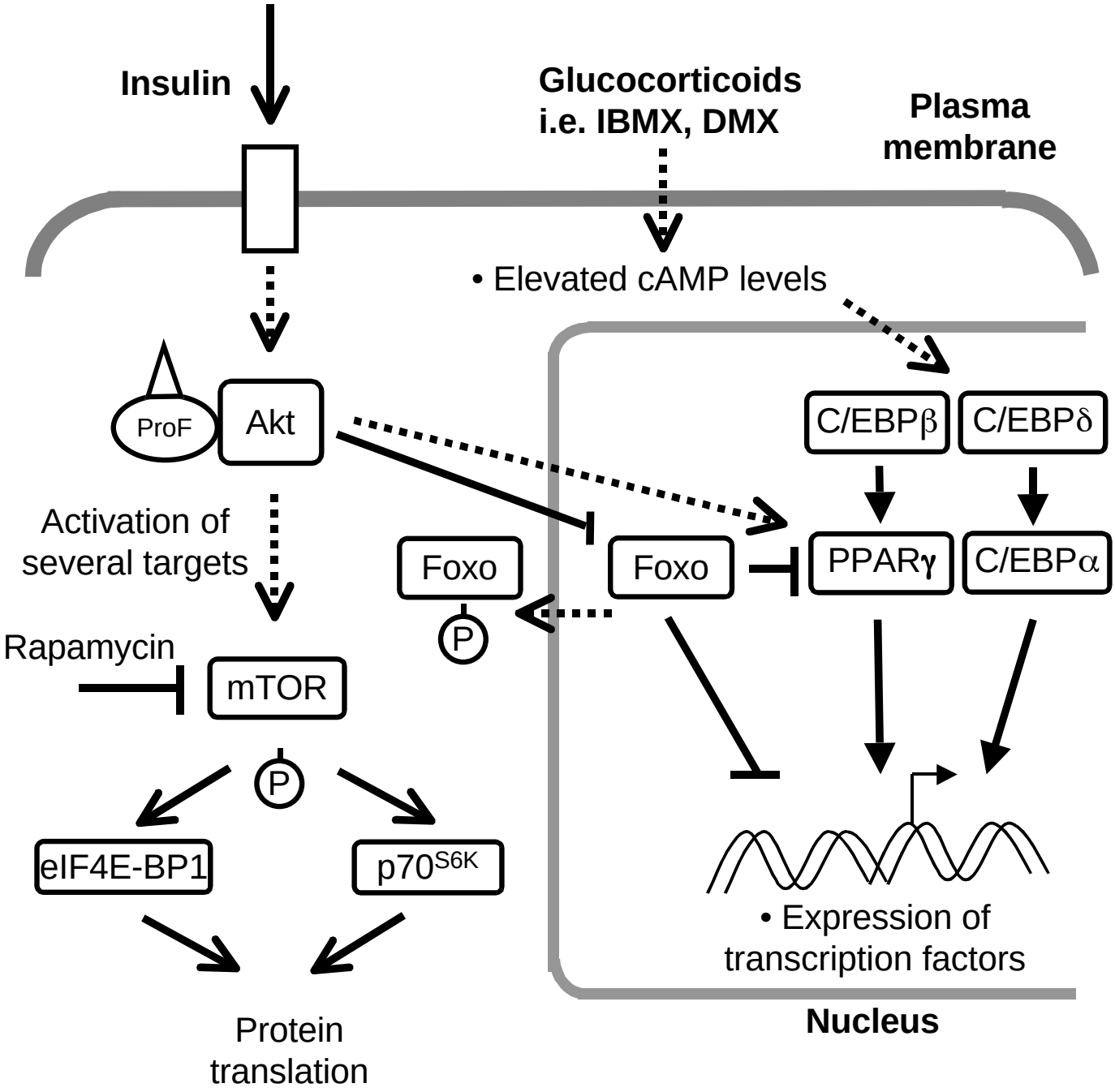
Day +1
Day +4
D
Day +1
Day +4
1
2
3
4
1
2
3
4

Figure 8


Insulin
Glucocorticoids
i.e. IBMX, DMX
Plasma
membrane
• Elevated cAMP levels
ProF
Akt
C/EBPβ
C/EBPδ
Activation of
several targets
Foxo
Foxo
PPARγ
C/EBPα
P
Rapamycin
mTOR
P
eIF4E-BP1
p70S6K
• Expression of
transcription factors
Protein
translation
Nucleus

5 Discussion

The topic of my thesis was to study a newly found propeller FYVE- protein, which we designated as ProF. Since the more specific aspects of this thesis have been covered in the manuscripts, the aim of this discussion is to integrate all results in order to obtain a general picture of the role of ProF in adipocytes.

In this PhD thesis, a new protein was characterized, which interacts with several proteins as demonstrated in preadipocytes and other cellular systems, such as brain tissue. Among these interaction partners are two kinases, Akt and PKCζ, and VAMP2, a protein involved in vesicular trafficking. Binding of these proteins is mediated by the WD-repeats of ProF. Indeed, WD-repeat proteins are known to coordinate the complex multiprotein assembly in many different cellular systems by specifically binding to one or more partner proteins (Li and Roberts, 2001; Smith et al., 1999).

The FYVE domain, the second structural motif on the ProF protein, is responsible for its interaction with cellular membranes by membrane lipid recognition. Several membrane-targeting domains have been identified in the last decade, however only FYVE domains were found to bind exclusively and with high specificity to PI3P (Stenmark and Aasland, 1999). Yet, a typical FYVE domain shows only a weak binding capacity towards PI3P due to its shallow PI3P-binding pocket and a single phosphate interaction. Therefore, most isolated FYVE domains are insufficient for membrane binding and require further interaction domains for efficient membrane recruitment (Dumas et al., 2001; Lawe et al., 2000). However, there is a small number of FYVE-containing proteins that, when overexpressed, translocate to endosomal membranes by themselves (Ridley et al., 2001; Seet and Hong, 2001; Tsukazaki et al., 1998). It should be noted that there is not one conserved motif within the FYVE domain that allows this higher binding affinity, but several individual deviations (Blatner et al., 2004). The FYVE domain of the protein FENS-1 is exceptional by showing the highest binding capacity of all FYVE domains investigated so far (Blatner et al., 2004; Ridley et al., 2001). FENS-1 is also interesting because it shows 60% sequence identity with ProF and a very similar domain structure. The sequence identity of the FYVE domain of ProF and FENS-1 is with 80% even higher than the overall sequence identity. This is not so surprising since the FYVE domain is

comparatively well conserved among all species (Stenmark et al., 2002), whereas the WD-repeats show very little sequence conservation among species (Li and Roberts, 2001). The structural feature that determines the high membrane affinity of the FYVE domain of FENS-1 is an 11-amino acid insertion next to the conserved (R/K)-(R/K-)H-H-C-R sequence, which is rich in aromatic amino acids and was found exclusively in the proteins ProF and FENS-1. Structure prediction analysis suggests that this motif forms an extended turret loop (Blatner et al., 2004). This exceptionally long hydrophobic protrusion has been shown to cause the high membrane penetration power and strong membrane affinity of the FYVE domain (Blatner et al., 2004). Deletion of the unique 11-amino acid insertion caused a dramatically (around 80-fold) decreased membrane affinity of the mutant compared with the wild type FENS-1. Furthermore, mutants of conventional FYVE domain proteins such as Hrs with an insertion of the FENS-1 motif exhibited an 11-fold higher membrane affinity than the wild-type protein. This clearly underscores the critical role of the hydrophobic insertion of FENS-1 in membrane binding.

Our data support this finding. Overexpressed ProF was found to localize to intracellular vesicles. This binding was clearly dependent on the FYVE domain binding to PI3P, because deletion of the FYVE domain and inhibition of PI3P-production by wortmannin led to loss of vesicular staining and to distribution of the protein in the cytoplasm. Similarly to FENS-1 (Blatner et al., 2004; Ridley et al., 2001), a single FYVE domain was sufficient for membrane localization of ProF. This demonstrates the high PI3P- binding capacity of ProF.

A key role of the phosphoinositide PI3P in the cell is the regulation of vesicular trafficking in the early endocytic pathway (Corvera, 2000) as well as in Golgi/ vacuole sorting (Odorizzi et al., 1998). Normally, PI3P is exclusively found at endosomes, where it is constitutively produced by the phosphatidylinositol kinase PI3K class III (Gillooly et al., 2001). More recently, it has been found that PI3P is also generated in a stimulus-dependent fashion (Razzini et al., 2000; Vieira et al., 2001; Zhang et al., 1998). One especially interesting aspect is the insulin-mediated formation of PI3P at the plasma membrane of adipocytes (Maffucci et al., 2003). This finding deserves a more in-depth discussion, because ProF plays a role in adipocytes and contains a FYVE domain for binding to PI3P.

As mentioned earlier, insulin activates class I PI3K, which produces PIP_2 and PIP_3, and which is required for insulin-dependent translocation of the glucose transporter GLUT4 (Cheatham et al., 1994; Tengholm and Meyer, 2002; Tsakiridis et al., 1995; Wang et al., 1999). In fat and muscle cells insulin also activates class II PI3K (Brown et al., 1999) to produce PI3P at the plasma membrane (Maffucci et al., 2003). To demonstrate the production of PI3P at this location Maffucci and coworkers transfected fat and muscle cells with plasmid constructs expressing a GFP-tagged tandem FYVE domain of Hrs. The GFP-FYVE fusion protein translocated to the plasma membrane upon insulin stimulation (Maffucci et al., 2003). This underscores the capability of adipocytes to recruit FYVE domain proteins to the plasma membrane after insulin stimulation. We found that the FYVE domain of ProF is capable of high-affinity binding to PI3P. Furthermore, our data showed that ProF partially translocated to the plasma membrane of adipocytes after insulin stimulation. This could be due to PI3P- production at this location.

After Maffucci and coworkers found insulin-induced PI3P-formation at the plasma membrane of fat and muscle cells, they wanted to know whether the generation of this phosphoinositide played a role in trafficking events. To investigate this possibility they decided to focus on the major trafficking event in adipocytes downstream of the insulin receptor: the translocation of GLUT4 from intracellular storage sites to the plasma membrane. To check whether the plasma membrane pool of PI3P was involved in this process, exogenous PI3P was targeted to the plasma membrane of fat and muscle cells via lipid carriers. Interestingly, PI3P at the plasma membrane led to the accumulation of GLUT4 vesicles close to the plasma membrane, but did not result in fusion of GLUT4 with the plasma membrane and, thus, did not cause increased glucose transport into the cells (Maffucci et al., 2003). These results were confirmed by overexpression of myotubularin, a PI3P phosphatase, which caused impaired insulin- induced GLUT4 translocation to the plasma membrane. This presumably happened because of reduced PI3P production in 3T3-L1 adipocytes upon insulin stimulation (Chaussade et al., 2003). Further studies showed that after PI3P- delivery the GLUT4 vesicles accumulated in a docked but unfused state close to the plasma membrane (Ishiki et al., 2005). Similar results were found when the cells were treated with inhibitors that inactivate class I PI3K, which produces PIP_3 to activate Akt2 and

PKCζ/λ, but do not inactivate class II PI3K, which produces PI3P. This was found both in fat (Bose et al., 2004) and muscle cells (Ishiki et al., 2005).

In summary, these results show that insulin- induced GLUT4 translocation is a two-step process. The first step consists of class I PI3K- independent accumulation of GLUT4 vesicles close to the plasma membrane (van Dam et al., 2005), which will be called GLUT4 plasma membrane accumulation from now on.

The second step in insulin-induced GLUT4 translocation is the fusion of the GLUT4 vesicles with the plasma membrane. This leads to the externalization of the glucose transporter, which allows the uptake of glucose into the cell. For clarity this part of the process will be called GLUT4 vesicle fusion from now on. The whole insulin-dependent process, which includes both GLUT4 plasma membrane accumulation and GLUT4 vesicle fusion, will be referred to as GLUT4 translocation.

The GLUT4 vesicle fusion step is dependent on class I PI3K, and probably also Akt2- and PKCζ/λ (Bose et al., 2004; Min et al., 1999). These data are in accordance with the report that activation of Akt2 and PKCζ/λ through class I PI3K-mediated production of PIP_3 is for the most part required at a late stage of insulin-dependent GLUT4 translocation and correlates with the GLUT4 vesicle fusion rather than GLUT4 plasma membrane accumulation (van Dam et al., 2005).

What does this mean for the adaptor protein ProF and its role in insulin-induced GLUT4 translocation? As mentioned before, ProF translocates to the plasma membrane upon insulin stimulation, possibly because of PI3P production at this location. Our experiments showed the translocation of ProF both in immunofluorescence studies and in cell fractionation experiments. Furthermore, we showed that Akt2 and PKCζ/λ translocated to the plasma membrane upon insulin stimulation (Fig. 6). Our latter finding is in accordance with numerous data from other groups (Braiman et al., 2001; Hanada et al., 2004; Kanzaki et al., 2004; Standaert et al., 1999).

One possible interpretation of our results is that ProF, which binds to PI3P, mediates the translocation of the kinases to the plasma membrane, where they can exert their role on GLUT4 vesicle fusion. A second possibility is that the kinases, which are activated by PIP_3 and PDK1 at the plasma membrane, recruit ProF to this location. In that case, ProF would bind to the activated kinases and PI3P at the plasma membrane might facilitate the recruitment. As a third possibility ProF and the kinases translocate

to the plasma membrane independently. In that case ProF would bind to PI3P, while the kinases would bind to PIP_3 and would be activated by PDK1. At this subcellular location ProF could preferentially bind to activated Akt2 and PKCζ/λ to integrate the kinases with their substrate proteins. At the moment we do not yet have the data to determine which one of these three possibilities is correct.

Regardless of the exact mechanism involved, all three proteins, ProF, Akt2, and PKCζ/λ translocate to the plasma membrane of adipocytes upon insulin stimulation. What could be the function of ProF at this location? As mentioned earlier, activated Akt2 and PKCζ/λ play a key role in GLUT4 vesicle fusion. Most probably they exert their function by phosphorylating target proteins that regulate docking and fusion of GLUT4 vesicles with the plasma membrane. At this step, ProF could bring together the kinases and their substrates by binding both proteins at the appropriate subcellular location.

What are these kinase substrates? Recently, several possible targets of Akt2 and PKCζ/λ involved in GLUT4 vesicle fusion have emerged. Many of these proteins appear to be involved in the regulation of the SNARE complex. In the process of insulin-dependent GLUT4 translocation, this SNARE complex consists of the t-SNAREs syntaxin 4 and SNAP-23 at the plasma membrane (Foster and Klip, 2000; Watson et al., 2004) and the v-SNARE VAMP2 on the GLUT4-containing vesicles (Martin et al., 1998), but it also includes numerous associated proteins (Grusovin and Macaulay, 2003). The SNARE complex formation by binding of VAMP2 to SNAP-23 and syntaxin 4 is crucial for fusion of GLUT4 vesicles with the plasma membrane. Akt and PKCζ/λ could either indirectly regulate the SNARE complex formation by phosphorylating regulators of SNARE proteins, or directly by phosphorylating the SNARE proteins themselves.

Recent results obtained from fat and muscle cells point to the fact that Akt2 and PKCζ/λ can indeed phosphorylate regulators of SNARE complex formation.

Akt2 appears to phosphorylate the protein Synip (Yamada et al., 2005), which is bound to the t-SNARE syntaxin 4 in unstimulated adipocytes to prevent it from binding to the v-SNARE VAMP2 (Foster and Klip, 2000; Min et al., 1999). In stimulated adipocytes, Synip dissociates from syntaxin 4 to allow SNARE-SNARE-interaction and GLUT4 vesicle fusion (Foster and Klip, 2000; Min et al., 1999). Results from Yamada and coworkers indicate that Akt2 can directly phosphorylate

Synip in vitro and that this phosphorylation decreases the binding of Synip to syntaxin 4 for GLUT4 vesicle fusion (Yamada et al., 2005), although these data have recently been questioned (Sano et al., 2005). Therefore, the exact role of Akt at the plasma membrane is still unclear.

One interesting but indirect target of the kinase PKCζ is the protein Munc18c, a binding partner for syntaxin 4 in adipocytes (Halachmi and Lev, 1996; Tellam et al., 1997; Tellam et al., 1995). Munc18c has been implicated in regulation of GLUT4 externalization, similar to Synip (Tamori et al., 1998; Tellam et al., 1997; Thurmond et al., 1998; Thurmond et al., 2000). The kinases Akt2 and PKCζ/λ appear to play an important role in Munc18c-dependent GLUT4 externalization. This has been recently demonstrated in adipocytes from Munc18c knock out mice, in which the action of PI3K-dependent kinases affected GLUT4 externalization in wild type, but not in Munc18c knock out mice (Kanda et al., 2005). This indicates that kinases downstream of PI3K – most likely Akt2 or PKCζ/λ - are involved in insulin-stimulated GLUT4 externalization and that one of their targets is the disruption of the syntaxin 4-Munc18c interaction. A recent publication shows that upon insulin stimulation PKCζ preferentially interacts with Munc18c in fat and muscle cells (Hodgkinson et al., 2005b). Furthermore, disruption of the PKCζ-Munc18c binding by overexpression of deletion mutants impaired insulin-stimulated GLUT4 translocation. However, Munc18c appears not to be a direct target of PKCζ (Hodgkinson et al., 2005b). It is possible that active PCKζ directly recruits another kinase to the plasma membrane. However, it is also conceivable that an adaptor protein is responsible for this recruitment. This protein could bind both, PKCζ and another kinase, at the correct subcellular location. ProF could be envisaged as such a protein, which allows the phosphorylation of regulators of SNARE protein interaction.

Furthermore, SNARE protein interaction leading to GLUT4 vesicle fusion could also be regulated by direct phosphorylation of the SNARE proteins. One publication by Braiman and coworkers showed evidence for such a direct SNARE phosphorylation involved in GLUT4 translocation to the plasma membrane (Braiman et al., 2001). Braiman and coworkers found that either insulin or overexpression of PKCζ induced serine phosphorylation of VAMP2. We confirmed and extended this knowledge by verifying the stimulation- and PKCζ-dependent phosphorylation of VAMP2. Furthermore, we found that the adaptor protein ProF binds to both proteins and forms

a ternary complex with PKCζ and VAMP2. Our in vitro studies also demonstrated that ProF increases the binding of PKCζ to VAMP2 and the PKCζ- dependent phosphorylation of VAMP2 (Fig.6). This underscores the role of ProF as adaptor protein. Additionally, it provides evidence that ProF promotes the phosphorylation of SNARE proteins by PKCζ and, thus, may play a role in the regulation of GLUT4 vesicle fusion to the plasma membrane.

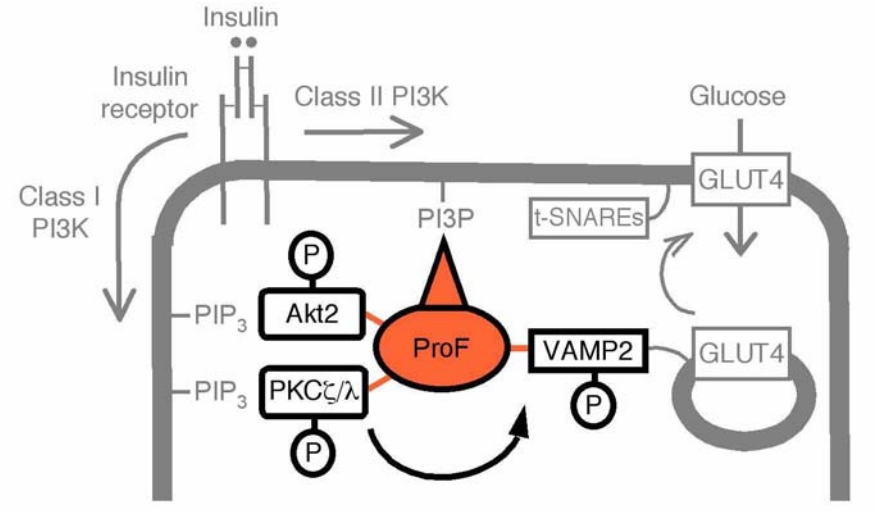


Fig. 6: Schematic model of the role of ProF insulin-dependent signaling events, which lead to plasma membrane translocation of GLUT4. Binding of insulin activates the insulin receptor, which stimulates class I PI3K (left arrow), leading to PIP_3 production at the plasma membrane, and class II PI3K (left arrow), leading to PI3P production at the plasma membrane. PIP_3 allows the activation of the kinases Akt2 and PKCζ/λ at the plasma membrane (left). ProF also translocates to the plasma membrane upon insulin stimulation, possibly via binding of the FYVE domain of ProF to PI3P (top). The model shows binding of ProF (center) to Akt2 and PKCζ/λ. Furthermore, ProF binds the SNARE protein VAMP2 (right), an in vitro substrate of PKCζ, which is located on GLUT4-containing vesicles. By interacting with both proteins, ProF increases VAMP2 substrate phosphorylation by PKCζ (black arrow) and might thereby regulate the GLUT4 vesicle fusion with the plasma membrane and glucose uptake (top, right). Proteins investigated in these studies are highlighted in black, other key proteins involved in plasma membrane translocation of GLUT4 are depicted in gray. For further details see text.

What could be the effect of ProF-mediated VAMP2 phosphorylation by PKCζ? We found that PKCζ can phosphorylate more than one serine residue on VAMP2 in vitro and that three of the four potential phosphorylation sites are within the SNARE motif of VAMP2. This motif binds to the SNARE-motifs of syntaxin 4 and SNAP-23 to allow the formation of a twisted, parallel four-helical bundle (Sutton et al., 1998). Because the formation of this helical bundle of SNARE motifs is dependent on hydrophobic interactions and because several highly polar phosphoserine residues could disturb the formation of the bundle, one would expect a negative effect of

VAMP2 phosphorylation on SNARE formation and GLUT4 vesicle fusion. However, several reports showed that decreased binding between v-SNARE and t-SNARE proteins could lead to an increased fusion of vesicles. For example, as discussed earlier, SNAP-25, the SNAP-23 homolog expressed in neurons, is phosphorylated by PKC at S187, which lies within the C-terminal SNARE motif (Gonelle-Gispert et al., 2002; Kataoka et al., 2000; Shimazaki et al., 1996). Unfortunately, all experiments were performed with a mixture of PKC isoforms or with PKC inhibitors, which exhibit little isoform specificity. Therefore it is not possible to distinct which PKC isoforms were responsible for the phosphorylation. Nevertheless, PKC-dependent phosphorylation of SNAP-25 caused decreased binding of SNAP-25 to syntaxin1 and increased neurotransmitter release, possibly by accelerating the SNARE complex dissociation and thus enhancing the efficiency of exocytosis (Shimazaki et al., 1996). More recently, similar results were obtained with syntaxin 4 and SNAP-23, the t-SNARE implicated in GLUT4 externalization (Foster and Klip, 2000; Watson et al., 2004). In the case of SNAP-23 it was found that stimulation of platelet cells caused PKC- dependent phosphorylation of syntaxin 4 at a so far undetermined position. This led to a decreased interaction of the protein with SNAP-23 and increased granule exocytosis (Chung et al., 2000). Likewise, PKC- mediated phosphorylation of SNAP-23 at S23 and T24 led to reduced syntaxin 4 binding and increased granule exocytosis (Polgar et al., 2003). In the case of syntaxin 4 it was suggested by Chung and coworkers that PKC- phosphorylation increased the exocytosis of alpha and dense granules by promoting disassembly of the SNARE complex. Thus, PKCζ-mediated phosphorylation of VAMP2 after stimulation may decrease the interaction of the v-SNARE protein VAMP2 with t-SNARE proteins. This may promote disassembly of the SNARE complex and thus may accelerate vesicle trafficking and glucose uptake as described for the SNARE proteins above. This hypothesis would be in accordance with the data from Kotani and coworkers, who showed that expression of constitutively active aPKC in adipocytes indeed promoted glucose transport (Kotani et al., 1998). We found that overexpression of ProF, which acts as adaptor for PKCζ and VAMP2 in vitro, also promotes glucose uptake in adipocytes. It is therefore possible that ProF might affect vesicle trafficking by increasing the PKCζ- mediated dissociation of the SNARE complex upon phosphorylation. It will be interesting to

test this hypothesis by analysis of t-SNARE association with PKCζ- phosphorylated or unphosphorylated VAMP2.

In summary, our results show that ProF mediates the binding of VAMP2 to PKCζ and the phosphorylation of VAMP2 by activated PKCζ, which could play a role in adipocytes, but possibly also in muscle cells and in a multitude of other cellular systems. Indeed, the process of SNARE-dependent fusion of vesicles with the plasma membrane upon stimulation is crucial not only for GLUT4 plasma membrane translocation, but also for many other vesicular trafficking systems, such as insulin release from pancreatic β-cells upon elevated glucose levels (Regazzi et al., 1995; Sadoul et al., 1995; Wheeler et al., 1996), zymogen granule release from pancreatic acinal cells upon stimulation (Wang et al., 2004), and synaptic vesicle exocytosis (Burgoyne and Morgan, 2003), including the constitutive cycling of several neuronal transmembrane proteins, which serve as ion channels (Bobanovic et al., 2002; Sheng and Lee, 2001; Wan et al., 1997). Furthermore, it includes secretory granule release in platelet cells upon stimulation by thrombin (Flaumenhaft et al., 1999; Reed, 2004) and plasma membrane translocation of the aquaporin transporter in kidney duct cells upon vasopressin stimulation (Gouraud et al., 2002; Nielsen et al., 2002) or of the H+-K+-ATPase proton transporter in gastric parietal cells upon stimulation by histamin or gastrin (Okamoto and Forte, 2001; Peng et al., 1997). Additionally, SNARE proteins have emerged as major players in numerous intracellular vesicle trafficking events, such as transport from endosomes to lysosomes or the trans-Golgi network (summarized in (Hong, 2005)). It is possible that ProF is also involved in a subset of those trafficking events. In summary, our results point to a possible role of ProF in GLUT4 vesicle fusion as binding partner of the kinases Akt and PKCζ/λ, although in vivo studies in adipocytes clearly would be needed to confirm this role.

The question arises if the kinase Akt2 and PKCζ/λ and the adaptor protein ProF also play a role in the first step of insulin-dependent glucose uptake, the GLUT4 plasma membrane accumulation.

Some data point to an involvement of the kinases in this first step. Expression of kinase-inactive Akt1 (Ducluzeau et al., 2002) or Akt2 (Chen et al., 2003) fused to GLUT4 prevented GLUT4 plasma membrane accumulation in response to insulin. Furthermore, two targets of Akt have been implicated in vesicle cycling upstream of

the GLUT4 vesicle fusion. The Akt substrate of 160 kDa (AS160) has been reported to regulate the translocation of GLUT4 vesicles from intracellular storage sites (Kane et al., 2002; Sano et al., 2003; Zeigerer et al., 2004), while the FYVE domain containing protein PIKfyve is implicated in vesicular sorting, and thereby in influencing the translocation of GLUT4 vesicles (Berwick et al., 2004). Additionally, some groups have shown a translocation of Akt2 (Calera et al., 1998; Kupriyanova and Kandror, 1999) and PKCζ/λ (Standaert et al., 1999) to internal vesicles directly after insulin stimulation before the accumulation of GLUT4 vesicles close to the plasma membrane, although these data have been questioned by other groups (Hill et al., 1999). Therefore, Akt2 and PKCζ/λ could as well play a role in GLUT4 plasma membrane accumulation.

ProF is a vesicular protein and interacts with active Akt and PKCζ/λ. Thus, ProF could potentially also be located on the insulin-responsive GLUT4 storage compartment vesicles, termed GSVs. Our fractionation data show that ProF is found in the low density microsomal (LDM) fraction of unstimulated adipocytes. The LDM fraction contains all small vesicles, including the GSVs, but also Golgi membranes, endosomes and other intracellular membranes (Piper et al., 1991). In unstimulated adipocytes, ProF is likely to be located on endosomes, which contain constitutive levels of PI3P (Gillooly et al., 2001). Our immunofluorescence pictures show a punctuate staining of overexpressed Myc-tagged ProF in adipocytes, however the resolution of the immunofluorescence data is not sufficient to detect a colocalization of ProF with GLUT4 on vesicles. Furthermore, around 50% of total GLUT4 is found on non-insulin-responsive vesicles, such as endosomes (Hah et al., 2002; Martin et al., 1996; Ramm et al., 2000; Zeigerer et al., 2002), while the remainder of GLUT4 is located in the GSV compartment. Therefore, even a colocalization of ProF with GLUT4 could not demonstrate that ProF is located in the highly insulin-responsible GSV pool. It is possible that ProF binds to GSVs by interacting with the protein VAMP2. This v-SNARE is an important component of the GSVs (Malide et al., 1997; Sevilla et al., 1997; Volchuk et al., 1994), but is also found on other intracellular vesicles (Hong, 2005). Interestingly, Maffucci and coworkers showed a translocation of overexpressed FYVE proteins from endosomes to the plasma membrane of adipocytes upon insulin stimulation. This is similar to the translocation of ProF from intracellular vesicles to the plasma membrane after treatment of the cells with insulin.

Thus, it is likely that these internal vesicles, on which ProF resides, are endosomes and not GSVs (Maffucci et al., 2003).

Furthermore, most data suggest that the second step in GLUT4 translocation – the GLUT4 vesicle fusion - likely plays the main role in the overall process. The first step – the GLUT4 plasma membrane accumulation - may play a more permissive role, perhaps by facilitating the efficient delivery of GSVs to the plasma membrane (van Dam et al., 2005). Additionally, most data indicate that Akt or PKCζ/λ exert their role predominantly at the level of GLUT4 vesicle fusion (Bose et al., 2004; Elmendorf et al., 1999; Thurmond et al., 1998; van Dam et al., 2005). Therefore, the role of ProF as adaptor for Akt and PKCζ in insulin-dependent glucose uptake is more likely to be found at the plasma membrane than in intracellular storage sites.

Interestingly, we found that ProF does not only influence GLUT4 translocation and glucose uptake in adipocytes, but also the process of adipogenesis, the generation of new adipocytes by differentiation of precursor cells. The efficient knock down of ProF by siRNA delayed both, protein and lipid droplet accumulation in preadipocytes. We used two different siRNAs targeted against ProF and found that complete downregulation of ProF strongly influenced glucose uptake and adipogenesis, whereas partial downregulation had much weaker effects. The two adipocyte-specific aspects, adipogenesis in undifferentiated preadipocytes and GLUT4 translocation in differentiated adipocytes, are closely related, as many signaling enzymes of the GLUT4 translocation such as insulin, PI3K and Akt play an important role in this pathway (Accili and Taylor, 1991; Arribas et al., 2003; Bluher et al., 2002; Bluher et al., 2004a; Bluher et al., 2004b; Cinti et al., 1998; Garofalo et al., 2003; Miki et al., 2001; Valverde et al., 1999). For example, overexpression of a constitutively active form of Akt induces adipocyte differentiation while Akt knockout impeded adipogenesis (Kohn et al., 1996; Peng et al., 2003). Similarly, expression of constitutively active Akt increased glucose uptake and GLUT4 translocation to the plasma membrane, while dominant-negative Akt resulted in inhibition of glucose uptake (Czech and Corvera, 1999; Kohn et al., 1998; Kohn et al., 1996). Since ProF acts as interaction partner for Akt, and may in that way play a role in GLUT4 translocation, it is conceivable that ProF also acts on adipogenesis as adaptor protein of Akt. ProF can also interact with activated PKCζ/λ, however evidence for a role of PKCζ/λ in adipocyte differentiation is limited (Lacasa et al., 1995). In summary, it

appears that PKCζ/λ does not play an important role during adipocyte differentiation. It is very much possible that ProF acts as a adaptor to bring Akt into close proximity of target proteins involved in adipogenesis, very similar to its proposed role in GLUT4 translocation. However, the molecular pathways downstream of Akt are not clearly understood (Kohn et al., 1996; Magun et al., 1996). One of the most interesting proteins in respect to ProF and Akt is the serine-threonine kinase mTOR (Sakaue et al., 1998; Scott et al., 1998), which is highly upregulated during adipocyte differentiation (Withers et al., 1997) and probably affects adipogenesis by activating protein translation (Isotani et al., 1999). This is in accordance with the finding that the increase of total protein levels during adipogenesis is delayed in cells with knock down of ProF by siRNA. mTOR is also interesting, because it is found predominantly on internal vesicles of mammalian cells (Withers et al., 1997), which appears to be similar to ProF. Therefore, ProF could use its FYVE domain as membrane anchor to bind to mTOR- containing vesicles and bring activated Akt into close proximity of its substrate. Most other targets of Akt implicated in adipogenesis are nuclear transcription factors such as Foxo1 (Nakae et al., 2003) or GATA2 (Menghini et al., 2005), which are negatively regulated by Akt phosphorylation to promote adipocyte differentiation. However, since Akt has to translocate to the nucleus to phosporylate its targets (Andjelkovic et al., 1997; Meier et al., 1997), the vesicular protein ProF is unlikely to play a role as adaptor in that respect.

Clearly, more data are needed to unravel the role of Akt and its adaptor protein ProF in adipogenesis. Likewise, it is apparent that more studies are required to understand GLUT4 translocation in its full complexity. In both cases, the most important question to answer is the identification of substrates of Akt and PKCζ in vivo and to clarify their role in the system of GLUT4 translocation or adipogenesis. The analysis of a membrane-localized adaptor protein that interacts with key kinases of GLUT4 translocation at their target subcellular localization may provide important insights into how these kinases are brought at their correct subcellular localization and how the substrate phosphorylation of their key targets is achieved and regulated. This is especially important considering the worldwide prevalence of disorders connected with insulin-dependent glucose uptake into fat and muscle cells, but also with adipogenesis that regulates fat cell formation in the organism (Feve, 2005). The most important among these diseases is the non-insulin-dependent diabetes mellitus type 2

(type 2 diabetes), which has reached epidemic proportions in the past decade. Indeed, numerous studies report that impaired GLUT4 translocation in fat and muscle cells may represent a primary defect leading to the development of type 2 diabetes (Kahn, 1998; Mora and Pessin, 2002; Mueckler, 2001; Saltiel, 2001; Saltiel and Kahn, 2001; Shepherd and Kahn, 1999). This disease is known to affect large numbers of people from all ethnic groups and at all social and economic levels throughout the world (Zimmet et al., 2001). At the present time it is estimated that 190 million people worldwide have diabetes and that this will increase to 320 million by 2025. This epidemic may even cause a decline in life expectancy in several nations, including the United States in the 21st century (Olshansky et al., 2005).

In summary, these data emphasize the necessity to investigate type 2 diabetes and the molecular mechanisms related to this disease in depth. For that a comprehensive analysis of the signaling pathways leading to insulin-dependent GLUT4 translocation in fat and muscle cells is essential. Furthermore, a detailed investigation of the molecular components that regulate these pathways is of great importance to understand the molecular mechanisms of glucose uptake and the related diseases.

In this thesis I characterized one such adaptor protein that plays a role in insulin-stimulated glucose uptake. The further investigation of ProF as connector of kinases and substrates might allow a better understanding of many important questions of GLUT4 translocation, including signaling pathways and their regulation. Furthermore, ProF may also be more generally involved in a variety of vesicular trafficking processes in adipocytes as well as in other tissues. For example ProF may also play a role in exocytotic or secretory pathways or the constitutive cycling of vesicles and their proteins from intracellular sites to the plasma membrane (summarized in (Royle and Murrell-Lagnado, 2003)). A similar role has been proposed for other vesicular proteins involved in GLUT4 translocation, namely for AS160 and PIKfyve (Welsh et al., 2005). Discerning the role of ProF in these trafficking processes will be a main focus of further study on this protein.

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7 Appendix

7.1 Abbreviations

AGC	PKA, PKG, and PKC family serine-threonine kinases
Akt	Oncogene causing AKR mouse thymoma
aPKC	atypical PKC
APS	Adaptor protein containing PH and SH2 domain
ASK	Apoptosis signal-regulating kinase
AS160	Akt substrate of 160 kDa
BAD	Bcl-XL-antagonist, causing cell death
Bcl	B-cell leukemia
bFGF	basic fibroblast growth factor
COP	coatomer protein
cPKC	conventional PKC
CTMP	carboxyl-terminal modulator protein
DAG	diacylglycerol
type 2 diabetes	non-insulin-dependent diabetes mellitus type 2
DL	direct lysate
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthetase
ErbB2	avian erythroblastic leukaemia viral oncogene homologue 2
FENS-1	FYVE domain protein localized to endosomes-1
FoxO	Forkhead box class O transcription factor
FYVE	domain identified in Fab1p, YOTB, VAC1p, and EEA1
GAP	GTPase-activating protein
GLUT	Glucose transporter
G-protein	trimeric GTP-binding protein
GS	glycogen synthase
GSK	glycogen synthase kinase
GSV	GLUT4 storage vesicle

IB	Immunoblot
IGF-I	insulin-like growth factor I
IKK	IκB kinase
IL	Interleukin
ILK	integrin-linked kinase
IP	Immunoprecipitation
IRS-1	insulin receptor substrate 1
IκB	inhibitor of NF-κB
JIP1	JNK interacting protein 1
JNK	c-Jun N-terminal kinase
LDM	Low density microsomal fraction
m/p-Akt1	myristoylated and palmitoylated Akt1
MAP kinase	mitogen-activated protein kinase
MAPKAP-K2	mitogen-activated protein kinase-activated protein kinase 2
MDM2	murine double minute 2
MLK	mixed lineage kinase
MSK1	mitogen- and stress-activated protein kinase 1
mTOR	mammalian target of rapamycin
NFκB	nuclear factor-κB
NGF	nerve growth factor
NPKC	novel PKC
OPCA	OPR/PC/AID motif
P70S6K	p70 S6 kinase
P70S6K	p70 S6 kinase
P90RSK	p90 ribosomal S6 kinase
PAK	p21 activated kinase
PAR-6	Partitioning defective homolog-6
PB1	Phox and Bem-1 domain
PDGF	platelet derived growth factor
PDK1	phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	phosphoinositide-3-kinase
PI3P	phosphatidylinositol-3-phosphate

PIKfyve	PI3P kinase containing a FYVE domain
PIP_2	phosphatidylinositol-(3,4)-bisphosphate
PIP_3	phosphatidylinositol-(3,4,5)-trisphosphate
$PI(3,5)P_2$	phosphatidylinositol-(3,4)-biphosphate
PKA	cAMP-dependent protein kinase
PKB	Protein kinase B
PKG	cyclic GMP-dependent kinase
PKG	cGMP-dependent protein kinase
ProF	Protein containing a WD-repeat propeller and a FYVE domain
PS	Pseudosubstrate
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RAC	Protein related to protein kinase A and protein kinase C
RACK	Receptor for activated C kinase
RD	Regulatory domain
SARA	Smad anchor for receptor activation
SH2	Src homology 2
SH3	Src homology 3
siRNA	small interfering RNA
SNARE	soluble N-ethylmaleimide–sensitive factor attachment protein receptor
SOS	son of sevenless
Tcl	T-cell leukemia
TGF	transforming growth factor
TNF	tumor necrosis factor
TSC	tuberous sclerosis complex
VAMP2	vesicle-associated membrane protein 2.
VEGF	Vascular endothelial growth factor
WD-repeat	repetitive motif containing a weakly conserved C-terminal Trp (W) -Asp (D) dipeptide
XIAP	X-linked inhibitor of apoptosis protein
YAP	Yes-associated protein
ZIP	PKC zeta-interacting protein

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7.3 Curriculum vitae

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WD-FYVE protein binds the kinases Akt and PKCζ/λ and regulates glucose uptake in adipocytes
(submitted)

WD-FYVE protein binds VAMP2 and PKCζ and increases PKCζ-dependent phosphorylation of VAMP2
(submitted)

WD-FYVE protein regultes differentiation of preadipocytes and glucose uptake in adipocytes
(to be submitted)

7.4 Statement of contribution

The contribution of the PhD student Thorsten Fritzius to the manuscripts was as follows:

Manuscript 1: WD-FYVE protein binds the kinases Akt and PKCζ/λ and regulates glucose uptake in adipocytes.
Contribution: Fig. 1, Fig. 2A, Fig. 2B, Fig. 3B, Fig. 4A, Fig. 4C, Fig. 5E; Fig. 6A, Fig. 6B. Wrote the manuscript together with Prof. Dr. Karin Mölling and Dr. Jochen Heinrich, by aid of an older manuscript written by Dr. Elvira Haas, Dr. Gabriela Burkard and Dr. Alexander D. Frey.

Manuscript 2: A WD-FYVE protein binds VAMP2 and PKCζ and increases PKCζ-dependent phosphorylation of VAMP2.
Contribution: Fig. 1B, Fig. 1D, Fig. 2A, Fig. 2B, Fig. 4A, Fig. 4B, Fig. 5. A. Wrote the manuscript together with Prof. Dr. Karin Mölling and Dr. Alexander D. Frey.

Manuscript 3: WD-FYVE protein regulates differentiation of pre-adipocytes and glucose uptake in adipocytes.
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